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(54) Title: COMPOSITIONS, METHODS AND KITS RELATING TO REMODELIN

(57) Abstract: The invention relates to novel nucleic acids encoding a mammalian adventitia inducible and bone expressed gene designated REMODEL, and proteins encoded thereby, whose expression is increased in certain diseases, disorders, or conditions, including, but not limited to, negative remodeling, arterial restenosis, vessel injury, ectopic ossification, fibrosis, and the like. REMODELIN also plays a role in cell-cell and cell-matrix adhesion, bone density, bone formation, dorsal closure, one mineralization, calcification/ossification, and is associated with *spina bifida*-like phenotype. In addition, the invention related to affecting REMODELIN expression by administration of TGF-ß and control of cellular gene expression using REMODELIN. The invention further relates to methods of treating and detecting these diseases, disorders or conditions, comprising modulating or detecting REMODELIN expression and/or production of REMODELIN.

TITLE OF INVENTION

Compositions, Methods and Kits Relating to REMODELIN

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of U.S. Patent Application No. 09/692,081, filed on October 19, 2000, from which it is entitled priority under 35 U.S.C. §120.

BACKGROUND OF THE INVENTION

The present invention relates to identifying novel processes involved in mediating arterial remodeling and formation of bone and cartilage.

Arterial stenosis with reduction in blood flow is a common problem in many vascular diseases. Several growth factors have been implicated in the mechanisms leading to vascular stenosis. For instance, fibroblast growth factor 2 (FGF-2) has been identified as an important factor in mediating proliferation of smooth muscle cells leading to intimal lesion formation. Furthermore, it has been demonstrated that arterial stenosis in response to angioplasty is largely due to negative remodeling as a result of adventitial fibrosis. As more fully set forth below, transforming growth factor beta (TGF-β) signaling has been demonstrated to play an important role in arterial stenosis in that, among other things, inhibition of TGF-β signaling using a soluble TGF-β receptor type II dramatically reduced lumen narrowing by decreasing negative remodeling and adventitial matrix deposition as well by decreasing neointima formation. These results indicate the crucial role of TGF-β signaling in arterial response to injury.

Vascular remodeling is a response of blood vessels to both physiological and pathological stimuli, leading to either vessel enlargement (positive remodeling) or shrinkage (negative remodeling). It has been demonstrated that neointimal proliferation or intimal mass following angioplasty shows little correlation with restenosis because of permanent changes in vascular geometry (Kakuta et al.,

1994, Circulation 89:2809-2815; Nunes et al., 1995, Arterioscler. Thromb. Vasc. Biol. 15:156-165). Negative remodeling has been shown to account for most of the restenosis process (Mintz et al., 1993, Circulation 88:1-654), and is now generally considered the predominant cause of restenosis. A successful therapeutic approach to restenosis, therefore, would target negative vascular remodeling.

Several growth factors have been implicated in the mechanisms leading to vascular stenosis, such as fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β). Specifically, cellular responses involving TGF-β in the adventitia have gained increased attention for their potential involvement in adventitial remodeling (Wilcox et al., 1996, Int. J. Radiat. Oncol. Biol. Phys. 36:789-796; Wilcox and Scott, 1996, Int. J. Cardiol. 54S:S21-35; Shi et al., 1996, Circulation 93:340-348). There is evidence that proliferative events occurring in the adventitia contribute to vascular remodeling and restenosis in response to vascular injury (Wilcox et al., 1996, Int. J. Radiat. Oncol. Biol. Phys. 36:789-796; Wilcox et al., 1997, Ann. N.Y. Acad. Sci. 811:437-447; Scott et al., 1996, Circulation 93:2178-2187). There is now general agreement that TGF-β is a potential factor in the adventitial remodeling process (Shi et al., 1996, Arterioscler. Thromb. Vasc. Biol. 16:1298-1305).

Although it is known that the TGF-β family of cytokines can have a variety of effects on vascular cells, very little is known about the role of this family of cytokines in vascular remodeling. TGF-β affects many functions including proliferation of smooth muscle cells (SMC) (Halloran et al., 1995, Am. J. Surg. 170:193-197). It has been demonstrated that inhibition of SMC proliferation by TGF-β1 occurs via extension of the G2 phase of the cell cycle (Grainger et al., 1994, Biochem. J. 299:227-235). In contrast, it has also been shown that inhibition of SMC proliferation by TGF-β1 is due to arrest in the late G1 phase of the cell cycle (Reddy and Howe, 1993, J. Cell Physiol. 156:48-55). SMC derived from atherosclerotic lesions responded to TGF-β1 with an increase in proliferation, and lower levels of TGF-β receptor II (TGF-βRII) have been implicated in the lack of inhibition by TGF-β1 in these cells (McCaffrey et al., 1995, J. Clin. Invest. 96:2667-2675).

Further studies have established that TGF-β1 stimulates SMC proliferation *in vitro*. Low doses of TGF-β1 stimulated SMC proliferation via platelet-derived growth factor (PDGF)-amino acid (AA)-dependent and PDGF-AA-independent mechanisms, while higher doses of TGF-β1 were inhibitory (Battegay et al., 1990, Cell 63:515-524; Stouffer and Owens, 1994, J. Clin. Invest. 93:2048-2055). Bifunctional effects of TGF-β1 in migration assays with SMC were also demonstrated (Koyama et al., 1990, Biochem. Biophys. Res. Commun. 169:725-729; Mii et al., 1993, Surgery 114:464-470).

TGF-β1 also plays a role in intimal lesion formation as indicated by a 5-7 fold induction of TGF-β1 mRNA in the balloon-injured rat carotid artery, with elevated levels of TGF-β1 mRNA persisting for 2 weeks (Majesky et al., 1991, J. Clin. Invest. 88:904-910). During the 2 week period, elevated TGF-β1 mRNA levels correlated with increases in mRNA expression of fibronectin and alpha-2 (I) and alpha-1 (III) collagens. These studies also demonstrated that infusion of recombinant TGF-β1 caused an increase in intimal SMC proliferation *in vivo* (*id.*).

Among clinically significant findings regarding the role of TGF-β signaling in arterial response to injury, it has been demonstrated that TGF-β1 mRNA expression in restenotic lesions compared to primary atherosclerotic lesions is increased (Nikol et al., 1992, J. Clin. Invest. 90:1582-1592). In the rat balloon injury model, treatment with TGF-β1 antibodies caused a small but significant reduction in neointima formation (Wolf et al., 1994, J. Clin. Invest. 93:1172-1178). Overexpression of TGF-β1 in the rat carotid artery by adenoviral gene transfer led to transient neointima formation with cartilaginous metaplasia that almost completely resolved within 8 weeks (Shulick et al., 1998, Proc. Natl. Acad. Sci. USA 95:6983-6988). Without wishing to be bound by any particular theory, TGF-β1 may also effect vascular tone since the factor was found to suppress nitric oxide synthase expression (Perella et al., 1996, J. Biol. Chem. 271:13776-13780) while at the same time inducing the vasoconstrictor endothelin in SMC *in vitro* (Kurihara et al., 1989, Biochem. Biophys. Res. Commun. 159:1435-1440). Further, TGF-β1 has been implicated in anti-apoptotic effects in SMC (Herbert and Carmeliet, 1997, FEBS Lett. 413:401-404).

Studies examining the expression of TGF-β ligand and TGF-β receptor (TGF-βR) mRNAs using reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that TGF-β1, TGF-β3, and TGF-βRII mRNA levels were increased in the media of the injured rat carotid artery (Ward et al., 1997, Arterioscler. Thromb. Vasc. Biol. 17:2461-2470) and expression of TGF-β2 and TGF-β3 were also reported in SMC of the lung vasculature (Khalil et al., 1996, Am. J. Respir. Cell Mol. Biol. 14:131-138; Pelton et al., 1991, Am. J. Respir. Cell Mol. Biol. 5:522-530). However, reduced levels of TGF-βRII were demonstrated in human atherosclerotic lesions (McCaffrey et al., 1995, J. Clin. Invest. 96:2667-2675). The three TGF-β ligands have overlapping functions and all of them induce expression of the alpha-1 (I), alpha-2 (I) and alpha-1 (III) chains of collagen (Bray et al., 1998, Hypertension 31:986-994).

The role of TGF-β isoforms in vascular repair processes was examined using a rat balloon catheter denudation model (Smith et al., 1999, Circ. Res. 84:1212-1222). Proliferating and quiescent SMC in denuded vessels expressed high levels of mRNA for TGF-β1, TGF-β2, and TGF-β3, and lower levels of TGF-βRII mRNA (Smith et al., 1999, Circ. Res. 84:1212-1222). The role of TGF-β signaling in the rat carotid artery balloon injury model was tested and it was shown that control vessels developed an extensive neointima and adventitial fibrosis with abundant collagen production. Vessels from animals injected with a recombinant soluble TGF-βRII (designated as "TGF-βR:Fc") revealed only little neointima formation and much less collagen deposition in the adventitia. The adventitia also contained significantly fewer cells, indicating that the proliferation of adventitial fibroblasts is mediated by TGF-β. Further, inhibition of TGF-β signaling with TGF-βR:Fc dramatically reduced lumen narrowing by decreasing negative remodeling and adventitial matrix deposition, as well as neointima formation.

TGF-β has been implicated in myofibroblastic transdifferentiation (Orlandi et al., 1994, Exp. Cell Res. 214:528-536; Desmouliere et al., 1993, J. Cell Biol. 122:103-111; Verbeek et al., 1994, Am. J. Pathol. 144:372-382), causing fibroblasts to transiently express smooth muscle α-actin (Darby et al., 1990, Lab. Invest. 63:21-29). The expression of smooth muscle α-actin in the carotid artery was

examined using immunostaining at 4 days after balloon denudation when proliferation of adventitial fibroblasts is rapid. Immunoreactive smooth muscle α-actin was either completely absent or markedly reduced in the outer adventitia of vessels from rats treated with TGF-βR:Fc compared to controls. This result demonstrated that the induction of smooth muscle α-actin expression by adventitial fibroblasts is at least in part mediated by TGF-β.

Morphometric analysis of the carotid arteries demonstrated significant increases in lumen area in all rats treated with TGF-βR:Fc with an approximate 88% increase with a dose of 2 mg/kg given every other day for 2 weeks. Further, a dose of 0.5 mg/kg every other day for 2 weeks caused nearly a 60% increase in lumen area despite the fact that intimal lesion formation was not affected by this dose. These results indicate that loss of lumen area is in large part due to negative remodeling and measurements of the perimeter of the neointima (IEL) and media (EEL) demonstrated that all doses of TGF-βR:Fc used in this study significantly inhibited the reduction in IEL and EEL.

The effect of TGF-βR:Fc on remodeling is highly relevant to the clinical situation of restenosis after angioplasty (Mintz et al., 1993, Circulation 88:1-654; Mintz et al., 1994, Circulation 90:1-24). Immunostaining with anti-human IgG antibody demonstrated that the TGF-βR:Fc primarily localized to the adventitia and neointima indicating that these are the predominant sites of TGF-β activity because TGF-βR:Fc binds only active TGF-β. One prominent effect of soluble TGF-βRII was the effect on collagen synthesis, which was particularly striking in the adventitia of Masson's trichrome stained sections. It was further found that the effects of TGF-βR:Fc on collagen expression by Northern blot analysis of RNA isolated from carotid arteries 4 days after injury were markedly reduced for collagen Type I and Type III, but Type XV was unaffected. No differences in levels of osteopontin, tropoelastin, or fibronectin mRNA were detected.

Taken together, the aforementioned findings identify the TGF-β isoforms as major factors mediating adventitial fibrosis and negative remodeling following vascular injury. Thus, genes whose expression is affected by TGF-β are

likely involved in such TGF-β associated processes, including arterial stenosis mediated by, *inter alia*, adventitial fibrosis and negative remodeling.

Many of the factors and processes involved in artery repair are shared in the development of bone and cartilage. Bone remodeling is a dynamic physiologic process by which bone mass is maintained or adjusted in response to appropriate stimuli (reviewed in Ducy et al., 2000, Science 289:1501-4.). This process consists of two phases, bone resorption by osteoclasts followed by bone deposition by osteoblasts. These events occur continually throughout the skeleton and understanding the factors that regulate this process are likely to have important implications for the treatment of common bone diseases such as osteoporosis. Many vascular injury related genes have been found that are known for their function in bone. These include osteopontin (Giachelli et al., 1993, J. Clin. Invest. 92:1686-1696.), alkaline phosphatase (ALP), bone morphogenic proteins (BMPs), and osteocalcin (Balica et al., 1997, Circulation 95:1954-60.; Bostrom et al., 1993, J. Clin. Invest. 91:1800-9.; Bostrom et al., 1995, Am. J. Cardiol. 75:88B-91B). Calcifications frequently also occur dystrophically in arteries affected by atherosclerosis and it is a characteristic finding in the medial sclerosis of Mönckeberg. Furthermore, matrix Gla protein deficient mice typically develop extensive vascular calcifications (Luo et al., 1997, Nature 386:78-81.). For a better understanding of potential common denominators between the events taking place in injured arteries or tissues and bone/cartilage development, a brief description of the cellular events is provided here.

Wound healing is characterized by the formation of granulation tissue from connective tissue surrounding the damaged area and its components are inflammatory cells, proliferating fibroblasts and myofibroblasts (smooth muscle α-actin positive), and a rich capillary network. Matrix protein synthesis is abundant and as the wound closes and evolves into a scar, there is a decrease in cellularity associated with the disappearance of myofibroblasts (Desmouliere et al., 1995Am. J. Pathol. 146:56-66.). This cell loss occurs via apopototic cell death (Desmouliere et al., 1995Am. J. Pathol. 146:56-66.). A similar course of events can be observed in the adventitia of balloon-injured arteries where the response to injury leads to negative remodeling as the predominant cause of restenosis. TGF-ß is a major factor mediating the remodeling

process in arteries in response to injury (Smith et al., 1999, Circ. Res. 84:1212-1222.). Inhibition of TGF-ß function with a soluble TGF-ß receptor II completely blocks the transdifferentiation of adventitial fibroblasts into myofibroblasts, demonstrating that this transdifferentiation process is at least in part mediated by TGF-ß (Smith et al., 1999, Circ. Res. 84:1212-1222.).

Chondrocytes participating in endochondral ossification undergo events that have some resemblance with wound healing. These include proliferation in the proliferative zone, followed by hypertrophy and subsequent cell death by apoptosis. The ingrowth of blood vessels in this process is critical as inhibition of vascular endothelial growth factor- (VEGF) mediated angiogenesis causes a decrease in the hypertrophic zone with reduced bone formation (Gerber et al., 1999 Nat. Med. 5:623-8.). During this differentiation process matrix protein synthesis is abundant and TGF-ß as well as BMP members have been shown to participate in it (Olsen et al., 2000, Annu. Rev. Cell Dev. Biol. 16:191-220.).

An important gene for chondrocyte differentiation was first recognized through the discovery of a mutation that is associated with a severe dwarfing syndrome now referred to as campomelic dysplasia (Foster et al., 1994, Nature 372:525-30.). This syndrome is characterized by malformation of long bones, vertebrae, pelvic and skull bones. The gene affected was *Sox9*, a member of the *Sox* gene family of transcription factors. During mouse development *Sox9* transcript expression peaks in cartilage primordia at 11.5 to 14.5 dpc (Ng et al., 1997, Dev. Biol. 183:108-21.) and like collagen II expression continues to be high in prechondrocytes and chondrocytes. In the growth plate of long bones, expression of the cartilage specific collagen II and *Sox9* are seen in resting and proliferating chondrocytes. In hypertrophic chondrocytes, collagen II is still expressed while *Sox9* is turned off. In the absence of *Sox9* no chondrocyte specific marker genes are expressed in *Sox9* null cells of mouse chimeras (Bi et al., 1999, Nat. Genet. 22:85-9.) and no differentiation of chondrocytes from the perichondrium takes place. While other *Sox* gene family members also participate in chondrocyte specific gene expression *Sox9* appears to play a dominant role in this process.

As part of the endochondral ossification process cartilage mass increases by proliferation of chondrocytes as well as deposition of cartilage matrix. Once cartilage is formed chondrocytes located in the central region undergo further maturation into hypertrophic chondrocytes. These chondrocytes are characterized by their withdrawal from the cell cycle and the expression of collagen X which serves as a marker gene for these hypertrophic chondrocytes. With the recruitment of blood vessels primary ossification centers are then formed. The matrix produced by the hypertrophic chondrocytes is subsequently degraded and replaced by trabecular bone synthesized by osteoblasts which are replacing hypertrophic chondrocytes undergoing apoptosis. Simultaneously, a collar of compact bone is formed by osteoblasts located in the perichondrium giving rise to the bone collar around the primary ossification centers. At either end of the cartilage secondary ossification centers originate from the growth plate where a coordinated process of chondrocyte proliferation, maturation, and apoptosis gives rise to longitudinal bone growth.

A member of the hedgehog family of genes, Indian hedgehog (*Ihh*), has been implicated as a key regulator of bone formation by promoting chondrocyte proliferation and inhibiting chondrocyte hypertrophy (Bitgood et al., 1995, Dev. Biol. 172:126-38.). The *Ihh* deficient mouse has a severe dwarfing syndrome due to the lack of endochondral bone formation (St-Jacques et al., 1999, Genes Dev. 13:2072-86.). Proliferation of chondrocytes is inhibited and chondrocyte maturation is disturbed in these mice. Of interest is the fact that a constitutively active receptor for the parathyroid hormone-related protein (PTHrP) under the control of the collagen IIalpha1 promoter can rescue the chondrocyte maturation phenotype of the *Ihh* deficient mouse. PTHrP synthesis is stimulated by *Ihh* expressed by cells in the perichondrium (Vortkamp et al., 1996, Science 273:613-22.) and there appears to be a feedback mechanism between these two ligands regulating the relative proportions of hypertrophic and proliferative chondrocytes. Conditions associated with both increased and decreased numbers of proliferative chondrocytes are associated with dwarfism as this results in decreased formation of hypertrophic chondrocytes in both cases.

How expression of *Ihh* in the proliferating chondrocytes is regulated is currently not well understood. However, signaling via the FGFR3 (Chen et al., 1999, J. Clin. Invest. 104:1517-25.; Naski et al., 1998, Development 125:4977-88.) and the PTHrP receptor may inhibit while BMPs may serve as inducers of *Ihh* expression (Pathi et al., 1999, Dev. Biol. 209:239-53.). It is of interest that *Ihh* deficiency has very little consequences for osteoblast differentiation occurring during intramembraneous ossification (St-Jacques et al., 1999, Genes Dev. 13:2072-86.).

The transcription factor *Cbfa1* has been identified as a crucial factor regulating osteoblast differentiation in both intramembraneous and endochondral ossification. In the absence of *Cbfa1*, mice develop no bone tissue although a normal cartilage skeleton is formed (Schiffrin, 1995, Cardiology 86 Suppl. 1:16-22.; Komori, et al., 199, Cell 89:755-64.; Otto et al., 1997, Cell 89:765-71.). This demonstrates that *Cbfa1* is not essential for chondrogenesis although in certain instances chondrocyte hypertrophy appears to require *Cbfa1*. During embryonic development, *Cbfa1* expression is restricted to cells destined to differentiate either into chondrocytes or osteoblasts (Schiffrin, 1995, Cardiology 86 Suppl. 1:16-22.). *Cbfa1* expression later becomes restricted to osteoblasts with only low levels seen also in hypertrophic chondrocytes (Kim et al., 1999, Mech. Dev. 80:159-70.).

It is likely that other transcription factors act downstream of *Cbfa1* since osteoblasts do not appear until 14.5 days post coitus (dpc) while *Cbfa1* expression is detectable as early as 10.5 dpc in mouse embryos. Homeobox genes like *Msx2*, *Hoxa-2* and *Bapx1* are likely upstream of *Cbfa1*, regulating its expression (Tribioli et al., 1997, Mech. Dev. 65:145-62.; Tribioli et al., 1999, Development. 126:5699-711.; Satokata et al., 2000, Nat. Genet. 24:391-5.; Gendron-Maguire et al., 1993, Cell 75:1317-31.; Kanzler et al., 1998, Development 125:2587-97.). Deficiency in the homeobox gene *Dlx5* in mice is associated with delayed intramembraneous ossification and to a lesser extent also with endochondral ossification (Simeone et al., 1994, Proc. Natl. Acad. Sci. U S A 91:2250-4.). However, *Cbfa1* expression is not affected by this deficiency, indicating that *Dlx5* is either downstream of *Cbfa1* or in a different signaling pathway. Downstream of *Cbfa1* are several bone matrix proteins. *Cbfa1* is essential for bone matrix production as several bone matrix proteins including

collagen I, osteocalcin (an osteoblast specific protein), bone sialoprotein, and alkaline phosphatase have binding sites for *Cbfa1* in their promoters (OSE2 binding site) (Schiffrin, 1995, Cardiology 86 Suppl. 1:16-22.). Several members of the bone morphogenic family (BMP) can induce *Cbfa1* expression in vitro while TGF-ß inhibits the expression of *Cbfa1* in osteoblasts in vitro and controls the levels of osteoblast differentiation in vivo (Schiffrin, 1995, Cardiology 86 Suppl. 1:16-22.).

The major structural components of cartilage and bone are collagens I and II with essential roles in providing mechanical strength. Several disease entities are associated with mutations in these types of collagen with phenotypes ranging from normal stature with osteoarthritis to lethality (Alliston et al., 2001, EMBO J 20:2254-72.; Mundlos et al., 1997, FASEB J. 11:227-33.). Collagens play important roles in wound and fracture healing and consequently inhibited collagen formation will impair the healing process. Excessive collagen formation is also of clinical significance in the form of fibrosis in a variety of organs and tissues. Among the many diseases associated with collagen mutations are osteogenesis imperfecta (OI; collagen I) with brittle bones, chondrodysplasias (collagen II), several subtypes of Ehlers-Danlos syndrome (EDS, collagen III and others), Alport syndrome (collagen IV) with nephritis, Bethlem myopathy (collagen VI), dystrophic epidermolysis bullosea (DEB; collagen VII) with separation of the epidermis from the dermis, and many others (for review (Spranger et al., 1994, Eur. J. Pediatr. 153:56-65.).

The common structural element in all collagens is a unique triple-helical conformation which can be identified by its repetitive $(G\text{-}X\text{-}Y)_n$ amino acid sequence pattern. The conformation is stabilized by glycine as every third residue. The most common mutations are single base substitutions that cause replacement of a single glycine (G) by another amino acid. This results in interruption of the $(G\text{-}X\text{-}Y)_n$ sequence pattern and defective folding occurs as a consequence (for review see Myllyharju et al., 2001, Ann Med. 33:7-21). Collagen synthesis involves a number of post-translational modifications and fibril forming collagens are first synthesized as procollagen molecules with propeptide extensions at both the N- and C- terminal ends.

The intracellular steps involved in the assembly of procollagen molecules from pro α-chains include cleavage of the signal peptide, hydroxylation of

certain proline and lysine residues, glycosylation of certain asparagine residues, association of the C propetides, and formation of interchain and intrachain disulfide bonds. Following association of the C propeptides a nucleus of the triple helix is formed in the C-terminal region and the triple helix is then propagated towards the N terminus in a zipper-like fashion. These C propeptides allow the tethering of the 3 chains thus promoting triple helix formation, a process that can also be performed by a transmembrane domain (Baum et al., 1999, Curr. Opin. Struct. Biol. 9:122-8). The hydroxylation of proline residues in the Y position creates a unique region of 5-6 consecutive G-X-hydroxyP(roline) triplets at the C terminus of the triple-helix domain which are thought to act as a nucleation site for bringing the 3 chains into the correct dihedral angles and for forming the correct interchain hydrogen bonds. Studies by Bulleid et al. (Bulleid et al., 1997, EMBO J. 16:6694-701) demonstrated that this hydroxyP rich region is required for nucleation but that 2 consecutive G-X-hydroxyP triplets at the C terminus may be sufficient. During their transport from the endoplasmic reticulum (ER) through the Golgi stacks lateral aggregation of the procollagen molecules and increased condensation of the aggregates occurs during this process resulting in granule formation for secretion. In the extracellular space the N and C propeptides are cleaved and self-assembly of the collagen molecules into fibrils occurs followed by covalent cross-linking of collagen molecules (reviewed in Myllyharju et al., 2001, Ann. Med. 33:7-21).

Less abundant collagens in cartilage are collagens IX, X, and XI where collagens IX and XI have predominantly regulatory functions in fibril assembly. Expression of collagen X is limited to hypertrophic chondrocytes in the endochondral ossification process (Alliston et al., 2001, EMBO J. 20:2254-72). Important cartilage proteoglycans include perlecan and aggrecan. The latter forms large complexes with hyaluronic acid stabilized via link protein (Bulleid et al., 1997, EMBO J. 16:6694-701). Mutations in both link protein as well as aggrecan are associated with multiple skeletal abnormalities (Watanabe et al., 1998, J. Biochem, (Tokyo) 124:687-93.; Watanabe et al., 1194, Nat. Genet. 7:154-7).

Effective treatment of bone/cartilage and vascular diseases is dependent on understanding the processes by which these systems develop. Arterial stenosis with

reduction in blood flow is a common problem in many vascular diseases and it is an important causal factor in the morbidity and mortality associated with these diseases. Despite the fact that various growth factors, especially TGF-β, have been implicated in arterial stenosis, very few factors involved in arterial stenosis have been identified and characterized. Nevertheless, the identification of such factors is crucial in the development of diagnostics and therapeutics for treatment of vascular diseases associated or mediated by arterial stenosis. Thus, there is long-felt need for the identification and characterization of factors associated with arterial stenosis. The present invention meets this need. Similarly, proper formation of bone and cartilage is essential to normal development, and the formation of collagen matrices is an integral part of this process. Mutations of collagen genes are among the most common resulting in skeletal abnormalities. While little is known about the factors that modulate collagen matrices, understanding this process is highly relevant to many human diseases involving virtually all organ systems. Thus, there is a long felt need for the identification and characterization of factors associated with bone formation. The present invention meets this need.

BRIEF SUMMARY OF THE INVENTION

The invention includes an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

In one aspect, the nucleic acid shares at least about 33% sequence identity with a nucleic acid encoding at least one of rat REMODELIN (SEQ ID NO:1), and a human REMODELIN (SEQ ID NO:3).

The invention also includes an isolated nucleic acid encoding a mammalian REMODELIN, wherein the amino acid sequence of the REMODELIN shares at least about 6% sequence identity with an amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

The invention further includes an isolated polypeptide comprising a mammalian REMODELIN.

In one aspect, the mammalian REMODELIN molecule shares at least about 6% sequence identity with an amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

The invention includes an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the nucleic acid further comprising a nucleic acid encoding a tag polypeptide covalently linked thereto.

In one aspect, the tag polypeptide is selected from the group consisting of a green fluorescent protein tag polypeptide, an influenza virus hemagglutinin tag polypeptide, a myc tag polypeptide, a glutathione-S-transferase tag polypeptide, a myc-pyruvate kinase tag polypeptide, a His6 tag polypeptide, a FLAG tag polypeptide, and a maltose binding protein tag polypeptide.

The invention includes an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the nucleic acid further comprising a nucleic acid specifying a promoter/regulatory sequence operably linked thereto.

The invention includes a vector comprising an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

In one aspect, the vector further comprises a nucleic acid specifying a promoter/regulatory sequence operably linked thereto.

The invention includes a recombinant cell comprising an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

The invention includes a recombinant cell comprising a vector comprising an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

The invention includes an isolated nucleic acid complementary to the an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the complementary nucleic acid being in an antisense orientation. In one aspect, nucleic acid shares at least about 33% identity with a nucleic acid complementary with a nucleic acid having the sequence of at least one of a rat REMODELIN molecule (SEQ ID NO:1), and a human REMODELIN molecule (SEQ ID NO:3).

The invention includes a recombinant cell comprising an isolated nucleic acid complementary to the an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the complementary nucleic acid being in an antisense orientation.

The invention includes an antibody that specifically binds with a mammalian REMODELIN molecule polypeptide, or a fragment thereof.

In one aspect, the antibody is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, and a synthetic antibody.

The invention includes a composition comprising an antibody that specifically binds with a mammalian REMODELIN molecule polypeptide, or a fragment thereof, and a pharmaceutically-acceptable carrier.

The invention includes a composition comprising an isolated nucleic acid complementary to the an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the complementary nucleic acid being in an antisense orientation, and a pharmaceutically-acceptable carrier.

The invention includes a composition comprising an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, and a pharmaceutically-acceptable carrier.

The invention includes a composition comprising an isolated polypeptide comprising a mammalian REMODELIN and a pharmaceutically-acceptable carrier.

The invention includes a transgenic non-human mammal comprising an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

The invention includes a method of treating a disease mediated by abnormal expression of a REMODELIN molecule in a human. The method comprises administering to a human patient afflicted with a disease mediated by abnormal expression of a REMODELIN molecule a REMODELIN molecule expression-inhibiting amount of a composition comprising an isolated nucleic acid complementary to the an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment

thereof, the complementary nucleic acid being in an antisense orientation, and a pharmaceutically-acceptable carrier.

In one aspect, the disease is selected from the group consisting of impaired wound healing, fibrosis of an organ, ectopic ossification, and hypertrophic scar formation.

The invention further includes a method of diagnosing arterial restenosis in a mammal. The method comprises obtaining a biological sample from the mammal, assessing the level of REMODELIN in the biological sample, and comparing the level of REMODELIN in the biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with arterial restenosis, wherein a higher level of REMODELIN in the biological sample from the mammal compared with the level of REMODELIN in the biological sample from the like mammal is an indication that the mammal is afflicted with arterial restenosis, thereby diagnosing arterial restenosis in the mammal.

In one aspect, the biological sample is selected from the group consisting of a blood vessel sample, and a damaged tissue sample.

The invention includes a method of diagnosing negative remodeling in a mammal. The method comprises obtaining a biological sample from the mammal, assessing the level of REMODELIN in the biological sample, and comparing the level of REMODELIN in the biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with negative remodeling, wherein a higher level of REMODELIN in the biological sample from the mammal compared with the level of REMODELIN in the biological sample from the like mammal is an indication that the mammal is afflicted with negative remodeling, thereby diagnosing negative remodeling in the mammal.

The invention includes a method of diagnosing fibrosis in a mammal. The method comprises obtaining a biological sample from the mammal, assessing the level of REMODELIN in the biological sample, and comparing the level of REMODELIN in the biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with fibrosis, wherein a higher level of REMODELIN in the biological sample from the mammal compared with the level

of REMODELIN in the biological sample from the like mammal is an indication that the mammal is afflicted with fibrosis, thereby diagnosing fibrosis in the mammal.

The invention includes a method of identifying a compound that affects expression of REMODELIN in a cell. The method comprises contacting a cell with a test compound and comparing the level of REMODELIN expression in the cell with the level of REMODELIN expression in an otherwise identical cell not contacted with the test compound, wherein a higher or lower level of REMODELIN expression in the cell contacted with the test compound compared with the level of REMODELIN expression in the otherwise identical cell not contacted with the test compound is an indication that the test compound affects expression of REMODELIN in a cell. In one aspect, the invention includes a compound identified by this method.

The invention includes a method of identifying a compound that reduces expression of REMODELIN in a cell. The method comprises contacting a cell with a test compound and comparing the level of REMODELIN expression in the cell with the level of REMODELIN expression in an otherwise identical cell not contacted with the test compound, wherein a lower level of REMODELIN expression in the cell contacted with the test compound compared with the level of REMODELIN expression in the otherwise identical cell not contacted with the test compound is an indication that the test compound reduces expression of REMODELIN in a cell. In one aspect, the invention includes a compound identified by this method.

The invention includes a method of identifying a compound that affects TGF-β signaling. The method comprises contacting a cell with a test compound and comparing the level of REMODELIN expression in the cell with the level of REMODELIN expression in an otherwise identical cell not contacted with the test compound, wherein a higher or lower level of REMODELIN expression in the cell contacted with the test compound compared with the level of REMODELIN expression in the otherwise identical cell not contacted with the test compound is an indication that the test compound affects TGF-β signaling in a cell.

The invention includes a kit for alleviating a disease mediated by abnormal expression of a REMODELIN in a human. The kit comprises a REMODELIN expression-inhibiting amount of a composition comprising an isolated

nucleic acid complementary to the an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, the complementary nucleic acid being in an antisense orientation, and a pharmaceutically-acceptable carrier, the kit further comprising an applicator, and an instructional material for the use thereof.

In one aspect, the disease is selected from the group consisting of negative remodeling, arterial restenosis, vessel injury, fibrosis.

The invention includes a kit for alleviating a disease mediated by abnormal expression of a REMODELIN in a human. The kit comprises a REMODELIN expression-inhibiting amount of an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, and a pharmaceutically-acceptable carrier. The kit further comprises an applicator, and an instructional material for the use thereof.

The invention includes a kit for treating a bone disease in a mammal. The kit comprises a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression. The kit further comprises an applicator, and an instructional material for the use thereof.

The invention includes a kit for treating a cartilage disease in a mammal. The kit comprises a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, the kit further comprising an applicator, and an instructional material for the use thereof.

The invention includes a kit for inhibiting tissue calcification. The kit comprises a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, the kit further comprising an applicator, and an instructional material for the use thereof.

In one aspect, the tissue calcification is calcification of a transplant.

In another aspect, the transplant is a heart valve transplant.

The invention includes a method of increasing REMODELIN expression in a mammal. The method comprises administering a REMODELIN expression increasing amount of TGF-β to the mammal, thereby increasing REMODELIN expression in the mammal.

The invention includes a method of reducing REMODELIN expression in a mammal. The method comprises administering a REMODELIN expression reducing amount of TGF-β receptor type II to the mammal, thereby inhibiting signaling via TGF-β receptor type II and reducing expression of REMODELIN in the mammal.

The invention includes a method of affecting cellular gene expression in a mammal. The method comprises administering a nucleic acid encoding REMODELIN to the mammal, thereby affecting cellular gene expression in the mammal.

In one aspect, the cellular gene is selected from the group consisting of TGF-β1, collagen IIIα1, osteopontin, biglycan, alkaline phosphatase, and bone morphogenic protein 4.

In another aspect, the expression of osteopontin is dependent on Cbfa1.

The invention includes a method of affecting cellular gene expression in a mammal. The method comprises administering a nucleic acid antisense to a nucleic acid encoding REMODELIN to the mammal, thereby affecting cellular gene expression in the mammal.

The invention includes a method of treating bone disease in a mammal in need of such treatment. The method comprises administering to a mammal afflicted with the bone disease a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, thereby inhibiting REMODELIN expression and treating the bone disease in the mammal.

In one aspect, the bone disease is osteogenesis imperfecta.

The invention includes a method of treating cartilage disease in a mammal in need of such treatment. The method comprises administering to a mammal afflicted with the cartilage disease a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, thereby inhibiting REMODELIN expression and treating the cartilage disease in the mammal.

In one aspect, the collagen disease is selected from the group consisting of osteogenesis imperfecta (OI), dystrophic epidermolysis bullosea (DEB), and Bethlem myopathy.

The invention includes a method of diagnosing a bone disease in a mammal. The method comprises obtaining a biological sample from the mammal, assessing the level of REMODELIN in the biological sample, and comparing the level of REMODELIN in the biological sample with the level of REMODELIN in a biological sample obtained from an otherwise identical mammal not afflicted with bone disease, wherein a higher level of REMODELIN in the biological sample from the mammal compared with the level of REMODELIN in the biological sample from the like mammal is an indication that the mammal is afflicted with bone disease, thereby diagnosing the bone disease in the mammal.

In one aspect, the bone disease is osteogenesis imperfecta.

The invention includes a method of diagnosing a collagen disease in a mammal. The method comprises obtaining a biological sample from the mammal, assessing the level of REMODELIN in the biological sample, and comparing the level of REMODELIN in the biological sample with the level of REMODELIN in a biological sample obtained from an otherwise identical mammal not afflicted with a collagen disease, wherein a higher level of REMODELIN in the biological sample from the mammal compared with the level of REMODELIN in the biological sample from the like mammal is an indication that the mammal is afflicted with a collagen disease, thereby diagnosing the collagen disease in the mammal.

In one aspect, the collagen disease is selected from the group consisting of osteogenesis imperfecta (OI), dystrophic epidermolysis bullosea (DEB), and Bethlem myopathy.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiment(s) which are presently preferred. It should be understood, however, that invention is not limited to the precise arrangements and instrumentalities shown. In the drawings:

Figure 1A-1 is an image of a Northern blot depicting total RNA isolated from various rat organs probed with labeled REMODELIN (REMODELIN) cDNA. A significant 1.2 kb band was present in cultured rat aortic smooth muscle cells, ballooned rat aorta, lung, and brain. Significantly lower REMODELIN mRNA levels were detected in other tissues (*i.e.*, liver, thymus, spleen, kidney, heart, muscle, uterus, and testis). A transcript of about 3.5 kb was detected in SMC upon longer exposure of the blot or loading of higher amount of RNA on the gel.

Figure 1A-2 is an image depicting the gel used for Northern blot analysis in Figure 1A-1. The gel was stained with ethidium bromide.

Figure 1B-1 is an image of a Northern blot depicting expression of REMODELIN mRNA in 8 day balloon-injured rat carotid arteries and normal carotid arteries. The data depicted herein demonstrate that REMODELIN mRNA is expressed in the injured arteries only.

Figure 1B-2 is an image depicting the gel used for Northern blot analysis in Figure 1B-1. The gel was stained with ethidium bromide.

Figure 1C-1 is an image of a Northern blot depicting levels of REMODELIN mRNA in MC3T3 cells. The data disclosed herein demonstrate that the levels of REMODELIN mRNA were increased by the addition of bone morphogenetic protein-4 (BMP-4), with peak expression after 8 hours.

Figure 1C-2 is an image of a Northern blot depicting levels of REMODELIN mRNA in MC3T3 cells. The data disclosed herein demonstrate that the levels of REMODELIN mRNA were increased by the addition of TGF-β, with peak expression after 8 hours.

Figure 1C-3 is an image depicting the gel used for Northern blot analysis in Figure 1C-2. The gel was stained with ethidium bromide.

Figure 2A is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (sequence, SEQ ID NO:6). The arrowheads indicate the position of the internal elastic lamina. The image depicts normal carotid arteries and demonstrates no detectable REMODELIN expression

therein. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2B is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The arrowheads indicate the position of the internal elastic lamina. The image demonstrates strong REMODELIN expression limited to the adventitia of 8 day balloon injured arteries. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2C is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The arrowheads indicate the position of the internal elastic lamina. The image demonstrates maintained but decreased REMODELIN expression in the adventitia two weeks post-injury. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2D is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The arrowheads indicate the position of the internal elastic lamina. The image demonstrates that at 4 weeks post-injury, expression levels of REMODELIN expression were similar to levels detected in normal, control vessels. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2E is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts a transverse section of an 11.5 days *post coitus* (dpc) mouse embryo wherein REMODELIN expression is detectable in the developing mesoderm. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2F is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (sequence, SEQ ID NO:6). The image depicts a 14.5 dpc mouse embryo expressing REMODELIN in developing bone. The developing brain and bone are depicted. The image depicts that REMODELIN

expression becomes limited to the developing bone at later stages of embryo development. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2G is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts REMODELIN expression in the bones of the snout in a 14.5 dpc embryo. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2H is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts strong detectable REMODELIN expression in the bone of the developing skull of a 14.5 dpc mouse embryo. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2I is an image depicting an *in situ* hybridization analysis using[^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts osteoblasts adjacent to mineralized bone in a femur from a rat pup depicting expression of REMODELIN mRNA. The arrowheads indicate the transition from the osteoblast layer to the mineralized bone layer at the upper right portion of the image. The image demonstrates strong REMODELIN expression in osteoblasts along mineralized bone. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2J is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts osteoblasts adjacent to mineralized bone in a femur from a rat pup depicting expression of REMODELIN mRNA. The arrowheads indicate the transition from the osteoblast layer to the mineralized bone layer at the upper right portion of the image. The image demonstrates strong REMODELIN expression in osteoblasts along mineralized bone. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2K is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image

depicts expression of REMODELIN was undetectable in normal skin (the skin surface is located on the left side of the image). The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 2L is an image depicting an *in situ* hybridization analysis using [^{35}S]-UTP labeled antisense REMODELIN riboprobe (SEQ ID NO:6). The image depicts extensive expression of REMODELIN in a 7 day old skin incision along the wound edge in (myo)fibroblasts of the granulation tissue. The presence of silver grains, appearing as white specks under dark field illumination, indicates REMODELIN expression.

Figure 3 is a diagram depicting the putative domains within the rat REMODELIN-short (REMODELINs) protein. The following domains are indicated: transmembrane domain/signal peptide (amino acid residues from about 1 to 32); a CK2 phosphorylation domain (amino acid residues from about 31 to 34); an N-myristoylation domain (amino acid residues from about 69 to 74); a CK2 phosphorylation domain (amino acid residues from about 99 to 102); an N-myristoylation domain (amino acid residues from about 119 to 124); a PKC phosphorylation domain (amino acid residues from about 146 to 148); an N-myristoylation domain (amino acid residues from about 165 to 170); an N-glycosylation domain (amino acid residues from about 188 to 191); a CK2 phosphorylation domain (amino acid residues from about 197 to 200); an N-myristoylation domain (amino acid residues from about 201 to 206); an N-myristoylation domain (amino acid residues from about 205 to 210); and a CK2 phosphorylation domain (amino acid residues from about 219 to 222).

Figure 4A is an image depicting the nucleic acid sequences for the rat (SEQ ID NO:1) and human (SEQ ID NO:3) REMODELIN cDNA. Sequence homology between rat and human REMODELIN cDNA is about 78% at the amino acid level. Translational start sites and stop codons are underlined. Gaps introduced into a sequence to maximize the alignment are indicated by a dash ("-").

Figure 4B is an image depicting a comparison of the amino acid sequences of rat (SEQ ID NO:2) and human (SEQ ID NO:4) REMODELIN. The data disclosed demonstrate that the two proteins share about 95% sequence identity. A

consensus sequence is depicted between the two sequences. The "+" indicates a conserved amino acid substitution whereas "-" indicates either a gap or non-conserved amino acid substitution.

Figure 4C is an image depicting the amino acid sequence of the long form of rat REMODELIN (rREMODELIN$_L$) (SEQ ID NO:5), encoded by the isolated nucleic acid SEQ ID NO:1 depicted in Figure 4A, *supra*.

Figure 5A is an image of an autoradiograph depicting expression of REMODELIN protein using a rabbit reticulocyte lysate expression system. The image depicts the proteins produced by *in vitro* translation using the long and short forms of REMODELIN cDNA. Using the long form of the rat REMODELIN cDNA that contains an additional 5' in frame AUG start codon as a template, a predominant 34 kDa protein was expressed and lesser amounts of a 30 kDa protein was detected. Only the 30 kDa protein was produced when translation was performed using the short form of REMODELIN cDNA.

Figure 5B is an image depicting NIH3T3 cells transfected with a myc-tagged REMODELIN (myc-REMODELIN) expression construct. The myc-REMODELIN fusion protein product was detected using anti-myc antibody using confocal microscopy. The image depicts that immunoreactivity was observed throughout the cytoplasm in a punctate/vesicular pattern. Nuclear counterstain was performed using propidium iodide.

Figure 5C is an image of an immunoblot probed using rabbit antibody raised by immunizing using the carboxyterminal 15 amino acid residues of REMODELIN (*i.e.*, anti-REMODELIN IgG). Cell lysates obtained from normal carotid arteries, and 1, 4, 7, 14 and 28 day balloon injured rat carotid arteries were resolved using SDS-PAGE and the proteins were transferred by Western blotting. The REMODELIN antibody recognized a single band of approximately 34 kDa band only in the cell lysate prepared from the injured vessel but not in the normal vessel (nor. carotid).

Figure 5D is an image of an immunoblot probed using rabbit anti-REMODELIN IgG demonstrating expression of REMODELIN protein in various cell lines from different species as follows: NIH3T3, bovine aortic epithelium (BAE),

PAC-1 (a rat smooth muscle cell line), Ar75 (a rat smooth muscle cell line), RASMC (rat aortic smooth muscle cells), 293 cells, BASMC (bovine aortic SMC), 10T1/2 cells, human umbilical vein endothelial cells (HUVEC), A431 cells, and human aortic SMC (HASMC).

Figure 5E is an image of an immunoblot probed using rabbit anti-REMODELIN IgG depicting the effect of TGF-β1 or soluble TGF-β receptor type II (sol. TGF-βRII) on REMODELIN expression. MC3T3 cells were treated with 1 ng/ml of TGF-β1 or 100 ng/ml TGF-βRII and the cells were harvested at the times indicated in the image. The data disclosed demonstrate that TGF-β1 stimulated REMODELIN expression while TGF-βRII inhibited REMODELIN expression. Approximately 30 micrograms of protein were loaded per lane.

Figure 5F is an image depicting BAE cells transfected with a myc-tagged REMODELIN (myc-REMODELIN) expression construct. BAE were transiently transfected with a myc-tagged REMODELIN expression construct. Expression of the transfected REMODELIN fusion protein was detected using an anti-myc antibody and the data disclosed demonstrate that very little expression is detectable at 48 hours post-transfection. Without wishing to be bound by any particular theory, these data suggest loss of the transfected cells. BAE transfected with an unrelated protein (EP1) using the same vector as that used to prepare the myc-tagged REMODELIN construct demonstrated higher levels of fusion protein expression 48 hours after transfection.

Figure 6 is a series of images of Northern blots hybridized with the indicated probes. RNA was isolated from NIH3T3 cells stably transfected with a REMODELIN expression vector and corresponding vector transfected cells. The antisense REMODELIN transfected MC3T3 cells and corresponding vector transfected cells are the same as shown in Figure 12. REMODELIN overexpressing cells showed reduced levels of TGF-β1, collagen III, and biglycan while in antisense transfected cells, these levels were elevated. Osteopontin and ALP were highly upregulated in the absence of REMODELIN expression while BMP-4 was down-regulated. An ethidium bromide stained membrane is shown as a loading control.

Figure 7 is a set of graphs depicting luciferase activity in NIH3T3 cells transiently transfected with luciferase reporter constructs. Cbfa1-dependent luciferase activity was completely inhibited in the presence of cotransfected remodelin. Luciferase activity under the control of the osteopontin promoter was inhibited in the presence of cotransfected remodelin.

Figure 8 is an image of an immunoblot of recombinant remodelin protein probed with an anti-His tag antibody.

Figure 9A is an image depicting the resulting phenotypes in *Xenopus* embryos after injection of REMODELIN mRNA at the oocyte 2 cell stage. At the 17-cell stage, embryos injected with *lacZ* control RNA (shown on the left side of the image) exhibited normal development while embryos injected with REMODELIN mRNA (shown on the right side of the image) exhibited inhibition of neurectodermal cell migration.

Figure 9B is an image depicting normal control embryos at the 34-cell stage.

Figure 9C is an image depicting REMODELIN-injected 34-cell stage embryos. The REMODELIN-injected embryos were smaller, distorted, and demonstrated abnormal development of the head compared with control embryos depicted in Figure 6B.

Figure 9D is an image depicting a REMODELIN-injected embryo exhibiting an unfused neurectoderm due to failure of the neural tissue cells to migrate.

Figure 9E is an image depicting a REMODELIN-injected embryo exhibiting displaying the split tail phenotype common in REMODELIN-injected embryos.

Figure 10 is an image depicting the nucleic acid sequence (SEQ ID NO:9) of a myc-tagged REMODELIN construct.

Figure 11 is an image of an immunoblot depicting remodelin protein levels in MC3T3-E1 cells stably transfected with control vector or full length rat antisense remodelin cDNA. Levels of remodelin protein in antisense transfectants were undetectable while levels were normal in vector transfectants. The lower band is non-specific.

Figure 12A is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with control vector and clonal populations were isolated. The data disclosed demonstrate that vector-transfected cells were of a cobblestone morphology. The image depicts a phase contrast image using 200X original magnification.

Figure 12B is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with control vector and clonal populations were isolated. The data disclosed demonstrate that vector-transfected cells were of a cobblestone morphology. The image depicts a phase contrast image using 200X original magnification.

Figure 12C is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with control vector and clonal populations were isolated. The data disclosed demonstrate that vector-transfected cells were of a cobblestone morphology. The image depicts a phase contrast image using 200X original magnification.

Figure 12D is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 12E is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 12F is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 12G is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 12H is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 12I is an image depicting the effect of REMODELIN on cell adhesion and cell-cell contacts. MC3T3 cells were stably transfected with full length rat antisense REMODELIN cDNA and clonal populations were isolated. The data disclosed herein demonstrate that antisense REMODELIN transfected cells exhibit a distinctly altered phenotype with less adhesion to the substratum and reduced cell-cell contacts compared with control vector transfected cells. The image further depicts the

increased number in dead cells and cell debris in the antisense transfected cells. The image depicts a phase contrast image using 200X original magnification.

Figure 13A is a graph demonstrating that REMODELIN expression is associated with and/or mediates increased cell turnover. MC3T3 cells were transfected with control vector or full-length rat antisense REMODELIN cDNA and clonal populations were isolated. Cells were harvested at the time points indicated and cell numbers were determined. The data disclosed demonstrate that there was no increase in cell number in the antisense transfected cells compared with control cells.

Figure 13B is a graph demonstrating that REMODELIN expression is associated with and/or mediates increased cell turnover. MC3T3 cells were transfected with control vector or full-length rat antisense REMODELIN cDNA and clonal populations were isolated. The cells were pulsed with [^{3}H]-thymidine for 4 hours before measuring incorporation of tritium in DNA. The cells were harvested in parallel at the time points after plating indicated and cell numbers were determined (Figure 9A). The data disclosed demonstrate that there was increased cell turnover since there was increased [^{3}H]-thymidine incorporation but there was no increase in cell number in the antisense transfected cells compared with control cells (Figure 9A).

Figure 14A is an image depicting one day old REMODELIN transgenic mouse pups. Transgenic mice expressing REMODELIN under the control of the cytomegalovirus (CMV) promoter/regulatory sequence were generated and a transgenic female was bred with a transgenic male giving rise to the pups depicted herein. All of the transgenic pups exhibited hemorrhaging in the hip and shoulder regions.

Figure 14B is an image depicting one day old REMODELIN transgenic mouse pups. Transgenic mice expressing REMODELIN under the control of the cytomegalovirus (CMV) promoter/regulatory sequence were generated and a transgenic female was bred with a transgenic male giving rise to the pups depicted herein. X-ray examination of the skeleton identified that all transgenic mice were smaller with considerable shortening of the long bones. The image of three transgenic ("transg.") and one normal mouse are depicted.

Figure 14C is an image depicting one day old REMODELIN transgenic mouse pups. Transgenic mice expressing REMODELIN under the control of the cytomegalovirus (CMV) promoter/regulatory sequence were generated and a transgenic female was bred with a transgenic male giving rise to the pups depicted herein. Without wishing to be bound by any particular theory, similar to a *spina bifida* phenotype, the transgenic mice exhibited protrusion of neural tissue through the dorsal muscle layers in the thoracic area.

Figure 15A is an image depicting one day old transgenic mouse pups expressing REMODELIN under the control of the CMV promoter. Breeding of a transgenic female with a transgenic male gave rise to the pups depicted. The arrowheads indicate hemorrhaging in the hip and shoulder regions.

Figure 15B is an image depicting one day old transgenic mouse pup expressing REMODELIN under the control of the CMV promoter. Breeding of a transgenic female with a transgenic male gave rise to the pup depicted. Skeletal preparations revealed fractures of the humerus.

Figure 15C is an image depicting one day old transgenic mouse pup expressing REMODELIN under the control of the CMV promoter. Breeding of a transgenic female with a transgenic male gave rise to the pup depicted. Skeletal preparations revealed fractures of the femur.

Figure 15D is an image depicting an age matched normal mouse. Alcian blue staining of skeletal preparations is shown.

Figure 15E is an image depicting one day old transgenic mouse pup expressing REMODELIN under the control of the CMV promoter. Breeding of a transgenic female with a transgenic male gave rise to the pup depicted. Alcian blue staining was absent from the intervertebral joints and the posterior portions of the vertebrae in REMODELIN transgenic mice compared to normal controls (see Figure 15D).

Figure 15F is an image depicting an age matched normal mouse. Cortical bone matrix of the tibia is shown.

Figure 15G is an image depicting one day old transgenic mouse pup expressing REMODELIN under the control of the CMV promoter. Breeding of a

transgenic female with a transgenic male gave rise to the pup depicted. Cortical bone matrix of the tibia (stained blue; shown in gray) was a markedly reduced in transgenics compared to controls (see Figure 15F).

Figure 15H is an image depicting a transgenic pup from a transgenic/wildtype cross with a more severe phenotype which died prenatally. Severe skeletal abnormalities and separation of the skin were evident.

Figure 15I is an image depicting a transgenic pup from a transgenic/wildtype cross with a more severe phenotype which died prenatally. X-ray analysis demonstrated dwarfism, a dramatic reduction in bone density, and malformations of all bones. (WT= wildtype; TG = REMODELIN transgenic).

Figure 15J is an image depicting a transgenic pup from a transgenic/wildtype cross with a more severe phenotype which died prenatally. Histologically, there was a considerable reduction in bone matrix (stained blue; shown in gray) in all bones including the skull.

Figure 15K is an image depicting a transgenic pup from a transgenic/wildtype cross with a more severe phenotype which died prenatally. Ribs had very little bone matrix.

Figure 15L is an image depicting a transgenic pup from a transgenic/wildtype cross with a more severe phenotype which died prenatally. Skin sections revealed separation of the epidermis from the dermis.

Figure 16A is an image depicting skeletal preparation made from one day old non-transgenic pups otherwise identical to REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray).

Figure 16B is an image depicting skeletal preparation made from one day old REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray). The image depicts reduced cartilage formation in all bones, including the distal phalanges of the feet when compared with normal, non-transgenic pups (Figure 11A).

Figure 16C is an image depicting skeletal preparation made from one day old non-transgenic pups otherwise identical to REMODELIN transgenic pups.

Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray).

Figure 16D is an image depicting skeletal preparation made from one day old REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray). The image depicts that cartilage was absent from the intervertebral joints and the posterior portions of the vertebra when compared with normal, non-transgenic pups (Figure 11C).

Figure 16E is an image depicting skeletal preparation made from one day old non-transgenic pups otherwise identical to REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray).

Figure 16F is an image depicting skeletal preparation made from one day old REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray). The image depicts that the anterior portions of the ribs, particularly the more caudal ones, exhibited a marked decrease in cartilage content when compared with normal, non-transgenic pups (Figure 11E).

Figure 16G is an image depicting skeletal preparation made from one day old non-transgenic pups otherwise identical to REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray).

Figure 16H is an image depicting skeletal preparation made from one day old REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray). The image depicts that the transgenic pups exhibited decreased bone density, particularly in the flat bones of the skull which had a transparent appearance, when compared with normal, non-transgenic pups (Figure 11G).

Figure 16I is an image depicting skeletal preparation made from one day old non-transgenic pups otherwise identical to REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray).

Figure 16J is an image depicting skeletal preparation made from one day old REMODELIN transgenic pups. Mineralized bone appears pink in color (darker gray) and cartilage appears blue (lighter gray). The image depicts that the transgenic pups exhibited decreased bone density compared with normal, non-transgenic pups (Figure 11I). The data disclosed demonstrate that decreased bone density is associated with fragility leading to multiple fractures such as a fractured humerus (arrow), which explain the hemorrhaging observed in upper and lower limbs (Figure 10A).

Figure 17A is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. In situ hybridization with antisense REMODELIN RNA shows mRNA expression in the cartilage of the humerus head.

Figure 17B is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. Immunostaining with anti-REMODELIN IgG on a longitudinal section of the tibia.

Figure 17C is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. Higher magnification of a tibia head stained for REMODELIN.

Figure 17D is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. REMODELIN mRNA is expressed in periosteal cells (PC) of the femur.

Figure 17E is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. Masson's Trichrome stained section depicting cortical bone of the tibia.

Figure 17F is an image depicting endogenous REMODELIN expression in normal one day old mouse pups. Similar section as shown in Figure 17E depicting some REMODELIN immunoreactivity in PC and osteoblasts (arrows) but not in osteocytes of the bone matrix (BM).

Figure 17G is an image depicting expression of the REMODELIN-myc transgene in mice. Immunostaining with the anti-myc antibody was performed on a section of the arm depicting the proximal radius.

Figure 17H is an image depicting expression of the REMODELIN-myc transgene in mice. High levels of the transgene proteins are expressed by osteocytes (arrowheads) and lower levels are expressed in osteoblasts.

Figure 17I is an image depicting expression of the REMODELIN-myc transgene in mice. Preimmune IgG showed little background on section of tibia cartilage.

Figure 18A is an image depicting one day old normal mice. Endogenous REMODELIN protein was not detectable in normal skin by immunostaining with anti-REMODELIN IgG.

Figure 18B is an image depicting one day old REMODELIN transgenic mice. High levels of the REMODELIN transgene were detected in the epidermis with anti-myc antibody and lower levels were detected in the dermis.

Figure 18C is an image depicting one day old REMODELIN transgenic mice. The connective tissue of the dermis appeared very loose (Trichrome stain).

Figure 18D is an image depicting one day old normal mice. Skeletal muscle in normal mice showed endogenous REMODELIN expression with anti-REMODELIN IgG immunostaining.

Figure 18E is an image depicting cross-sectioned skeletal muscle fibers from one day old normal mice.

Figure 18F is an image depicting longitudinally-sectioned skeletal muscle fibers from one day old normal mice.

Figure 18G is an image depicting cross-sectioned skeletal muscle fibers from one day old REMODELIN transgenic mice. The transgene specific REMODELIN-myc antibody demonstrated high levels of immunoreactivity in skeletal muscle of transgenic mice and many of the fiber bundles were hollow (arrowheads).

Figure 18H is an image depicting cross-sectioned skeletal muscle fibers from one day old REMODELIN transgenic mice. Muscle fibers of transgenic mice formed circular structures (arrowheads).

Figure 18I is an image depicting cross-sectioned skeletal muscle fibers from one day old REMODELIN transgenic mice. Muscle fibers of transgenic mice were less densely packed.

Figure 19 is an image depicting the sequence of an isolated REMODELIN antisense ribonucleic acid (SEQ ID NO:6) complementary to a portion of a nucleic acid encoding REMODELIN.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the discovery of a novel nucleic acid encoding a mammalian adventitia-inducible bone expressed molecule termed REMODELIN, previously referred to as REMODEL and/or adventitia induced bone expressed molecule (AIBE), and the proteins encoded thereby. The data disclosed herein demonstrate that REMODELIN plays a role in, *inter alia*, arterial restenosis mediated by or associated with adventitial fibrosis. As described more fully below, REMODELIN also plays a role in bone and cartilage formation. Identification of REMODELIN has important implications in the development of therapeutics and diagnostics for, among other things, adventitial fibrosis, arterial restenosis, negative remodeling, restenosis due to wound healing, and anti-cancer therapy.

More specifically, nucleic acids encoding REMODELIN have been isolated in both rat and human. These sequences are provided herein, and have no significant homology to any known cDNA sequence.

The data disclosed herein demonstrate that expression of REMODELIN is induced by vessel injury in mammals. That is, REMODELIN was expressed in balloon-injured rat carotid arteries but not in normal, uninjured vessels. Furthermore, REMODELIN was expressed selectively in the adventitia of the injured vessel, and was not expressed in the neointima or in the adventitia of normal vessels. Moreover, REMODELIN expression was induced by TGF-β. This is important since proliferative events occurring in the adventitia contribute to vascular remodeling and restenosis in response to vascular injury and recent data demonstrate that TGF-β is a factor in this adventitial remodeling process. Thus, these data further indicate that REMODELIN plays a role in cell proliferation and/or migration associated with vessel injury and restenosis due to negative remodeling.

The data disclosed herein also demonstrate that REMODELIN plays an important role in development of bone during mammalian embryogenesis.

REMODELIN is normally expressed during mouse embryogenesis, but expression is localized to developing bone. However, in the adult mouse, REMODELIN expression is virtually undetectable, expressing at very low levels in the adult brain and lung tissue.

Additionally, the data disclosed herein demonstrate that in studies using frog embryos, REMODELIN also plays a role in cell proliferation and/or migration in that expression of REMODELIN in frog embryos resulted in inhibition blastopore closure, failure of closure of the neural folds, formation of a split tail, and other developmental abnormalities. The REMODELIN-injected embryos also presented with decreased size and distortion and abnormal development of the head.

Injection of REMODELIN mRNA into frog embryos inhibited FGF-induced mesoderm formation. That is, animal caps from REMODELIN-injected embryos incubated with FGF-1 resembled animal caps incubated in the absence of FGF-1. Indeed, the data disclosed herein demonstrate that the phenotype observed in frog embryos injected with REMODELIN mRNA is similar to that of embryos injected from mRNA for dominant-negative FGF receptor constructs. These results, in addition to the induction of REMODELIN by TGF-β, further indicate that REMODELIN is an important factor in cell proliferation, migration, or both.

Additionally, over-expression of REMODELIN in transgenic mice gave rise to *spina bifida*-like spinal defects. The transgenic mouse pups exhibited altered bone density and bone growth further indicating that REMODELIN plays an important role in embryogenesis, including, but not limited to, a role in bone growth and dorsal closure.

The data disclosed herein also demonstrate that REMODELIN is localized in the cell membrane via five potential N-myristoylation sites. Without wishing to be bound by any particular theory, these myristoylation sites may serve to anchor REMODELIN protein in the cell membrane. This would indicate that REMODELIN is not a secreted protein, but rather, it is associated with the cell in mediating its effect(s).

In sum, the data disclosed herein demonstrate that REMODELIN plays a role in cell proliferation and/or migration and is involved in cellular signaling.

Furthermore, the data demonstrate that REMODELIN likely plays a role in adventitial fibrosis, negative remodeling and arterial restenosis, mediated by, among other things, smooth muscle cell proliferation. Therefore, the instant invention provides an *in vitro* model for the study of the function and role(s) of REMODELIN in arterial remodeling, adventitial fibrosis, and restenosis in vessels, as well as potential therapeutics and diagnostics for treatment of diseases, disorders or conditions associated with adventitial fibrosis, arterial restenosis, bone density and bone growth.

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

By the term "abnormal expression of REMODELIN," as used herein, is meant that the level of expression of a REMODELIN molecule (*e.g.*, rat $REMODELIN_S$, rat $REMODELIN_L$, human REMODELIN) in a cell is detectably higher or lower than the level of expression of REMODELIN in an otherwise identical cell where the otherwise identical cell is obtained from normal tissue that does not exhibit any detectable disease, disorder or condition associated with or mediated by expression of REMODELIN, such as, but not limited to, adventitial remodeling, adventitial fibrosis, arterial restenosis, negative remodeling, bone growth, bone fracture healing, wound healing in any tissue, and the like.

As used herein, the term "adjacent" is used to refer to nucleotide sequences which are directly attached to one another, having no intervening nucleotides. By way of example, the pentanucleotide 5'-AAAAA-3' is adjacent the trinucleotide 5'-TTT-3' when the two are connected thus: 5'-AAAAATTT-3' or 5'-TTTAAAAA-3', but not when the two are connected thus: 5'-AAAAACTTT-3'.

As used herein, amino acids are represented by the full name thereof, by the three letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following table:

Full Name	Three-Letter Code	One-Letter Code
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Tyrosine	Tyr	Y
Cysteine	Cys	C
Asparagine	Asn	N
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W

By the term "adventitial fibrosis," as used herein, is meant the extensive fibrous (connective) tissue formation in the outer layer (*i.e.*, adventitia) of a blood vessel. Adventitial fibrosis is associated with abundant deposition of extracellular matrix and proliferation of myofibroblasts and fibroblasts.

As used herein, to "alleviate" a disease, disorder or condition means reducing the severity of one or more symptoms of the disease, disorder or condition. This can include, but is not limited to, reducing the level of REMODELIN expressed in a cell or tissue (*e.g.*, smooth muscle cell, lung tissue, an artery), reducing the level of cell proliferation and or migration, affecting wound healing, affecting granulation

tissue formation, affecting bone growth and/or fracture healing, reducing negative remodeling, arterial restenosis and/or adventitial fibrosis, inhibiting premature calcification/ossification, inhibiting calcification of an implant (*e.g.* a heart valve), reducing or increasing the level of REMODELIN in a patient, compared with the level of REMODELIN in the patient prior to or in the absence of the method of treatment, and the like.

"Antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

By the term "applicator" as the term is used herein, is meant any device including, but not limited to, a hypodermic syringe, a pipette, a bronchoscope, a nebulizer, and the like, for administering the REMODELIN nucleic acid, protein, and/or composition of the invention to a mammal.

"Arterial restenosis," as that term is used herein, means the re-narrowing of an artery in response to a vascular intervention aimed at dilating a stenosed (*i.e.*, narrowed) artery.

"Biological sample," as that term is used herein, means a sample obtained from an animal that can be used to assess the level of expression of a REMODELIN, the level of REMODELIN protein present, or both. Such a sample includes, but is not limited to, a blood vessel (*e.g.*, carotid artery, aorta, and the like) sample, a lung tissue sample, a SMC sample, and a sample from any tissue undergoing wound healing.

By "candidate anti-REMODELIN drug," as the term is used herein, is meant a compound that when contacted with a cell, reduces the level of expression of a nucleic acid encoding a REMODELIN protein in the cell compared with the level of

REMODELIN expression in that cell prior to contacting the cell with the compound or which reduces the level of expression in the cell compared with the level of REMODELIN expression in an otherwise identical cell which is not contacted with the compound.

A "cartilage disease," is any disease, disorder or condition associated with, or mediated by, abnormal cartilage formation, modeling, and the like, compared to cartilage that is known to not be diseased. Typically, cartilage diseases are mediated by a mutation in a collagen gene resulting in misfolding of the resultant mutant collagen protein compared to the normal protein, thereby resulting in impaired function.

By "complementary to a portion or all of the nucleic acid encoding REMODELIN" is meant a sequence of nucleic acid which does not encode a REMODELIN protein. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the nucleic acid encoding a REMODELIN protein and thus, does not encode REMODELIN protein.

The terms "complementary" and "antisense" as used herein, are not entirely synonymous. "Antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand.

"Complementary" as used herein refers to the broad concept of subunit sequence complementarity between two nucleic acids, *e.g.*, two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (*e.g.*, A:T and G:C nucleotide pairs). As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory sequences specified on

the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

A "coding region" of a gene consists of the nucleotide residues of the coding strand of the gene and the nucleotides of the non-coding strand of the gene which are homologous with or complementary to, respectively, the coding region of an mRNA molecule which is produced by transcription of the gene.

A "coding region" of an mRNA molecule also consists of the nucleotide residues of the mRNA molecule which are matched with an anticodon region of a transfer RNA molecule during translation of the mRNA molecule or which encode a stop codon. The coding region may thus include nucleotide residues corresponding to amino acid residues which are not present in the mature protein encoded by the mRNA molecule (*e.g.*, amino acid residues in a protein export signal sequence).

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the

host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

A first region of an oligonucleotide "flanks" a second region of the oligonucleotide if the two regions are adjacent one another or if the two regions are separated by no more than about 1000 nucleotide residues, and preferably no more than about 100 nucleotide residues.

As used herein, the term "fragment" as applied to a nucleic acid, may ordinarily be at least about 20 nucleotides in length, preferably, at least about 50 nucleotides, more typically, from about 50 to about 100 nucleotides, preferably, at least about 100 to about 200 nucleotides, even more preferably, at least about 200 nucleotides to about 300 nucleotides, yet even more preferably, at least about 300 to about 350, even more preferably, at least about 350 nucleotides to about 500 nucleotides, yet even more preferably, at least about 500 to about 600, even more preferably, at least about 600 nucleotides to about 650 nucleotides, yet even more preferably, at least about 650 to about 800, more preferably, from about 800 to about 1000 nucleotides, preferably, at least about 1000 to about 1100 nucleotides, even more preferably, at least about 1100 nucleotides to about 1200 nucleotides, yet even more preferably, at least about 1200 to about 1210, even more preferably, at least about 1210 nucleotides to about 1220 nucleotides, yet even more preferably, at least about 1220 to about 1225, and most preferably, the nucleic acid fragment will be greater than about 1230 nucleotides in length.

However, as applied to a nucleic acid, the term fragment as used herein does not encompass the following isolated nucleic acids as referred to by their GenBank Accession numbers: AA335862 (sharing about 87% identity over about 373 nucleotides with REMODELIN cDNA); C01758 (sharing about 87% identity over about 356 nucleotides with REMODELIN cDNA); AA335551 (sharing about 87% identity over about 334 nucleotides with REMODELIN cDNA); AA406425 (sharing about 88% identity over about 312 nucleotides with REMODELIN cDNA); R46857; AA584310; D79314; AI085616; D62262; AA482398; AA482544; AI359844;

AI352209; AI239604; AI218433; AI081084; AI074870; AI074769; AA974239; AA969841; AA857920; AA723450; AA410434; AA738416; AI370649; AA507081.

As applied to a protein, a "fragment" of REMODELIN is about 20 amino acids in length. More preferably, the fragment of a REMODELIN is about 30 amino acids, even more preferably, at least about 40, yet more preferably, at least about 60, even more preferably, at least about 80, yet more preferably, at least about 100, even more preferably, about 100, and more preferably, at least about 150, more preferably, at least about 200, yet more preferably, at least about 240, even more preferably, at least about 243, yet more preferably, at least about 250, even more preferably, about 270, and more preferably, at least about 277 amino acids in length amino acids in length.

A "genomic DNA" is a DNA strand which has a nucleotide sequence homologous with a gene. By way of example, both a fragment of a chromosome and a cDNA derived by reverse transcription of a mammalian mRNA are genomic DNAs.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGGC share 50% homology.

As used herein, "homology" is used synonymously with "identity."

In addition, when the terms "homology" or "identity" are used herein to refer to the nucleic acids and proteins, it should be construed to be applied to homology or identity at both the nucleic acid and the amino acid sequence levels.

A first oligonucleotide anneals with a second oligonucleotide with "high stringency" or "under high stringency conditions" if the two oligonucleotides anneal under conditions whereby only oligonucleotides which are at least about 60%, more preferably at least about 65%, even more preferably at least about 70%, yet more preferably at least about 80%, and preferably at least about 90% or, more preferably, at least about 95% complementary anneal with one another. The stringency of conditions used to anneal two oligonucleotides is a function of, among other factors, temperature, ionic strength of the annealing medium, the incubation period, the length of the oligonucleotides, the G-C content of the oligonucleotides, and the expected degree of non-homology between the two oligonucleotides, if known. Methods of adjusting the stringency of annealing conditions are known (*see, e.g.,* Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

The determination of percent identity between two nucleotide or amino acid sequences can be accomplished using a mathematical algorithm. For example, a mathematical algorithm useful for comparing two sequences is the algorithm of Karlin and Altschul (1990, Proc. Natl. Acad. Sci. USA 87:2264-2268), modified as in Karlin and Altschul (1993, Proc. Natl. Acad. Sci. USA 90:5873-5877). This algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (1990, J. Mol. Biol. 215:403-410), and can be accessed, for example, at the National Center for Biotechnology Information (NCBI) world wide web site having the universal resource locator "http://www.ncbi.nlm.nih.gov/BLAST/". BLAST nucleotide searches can be performed with the NBLAST program (designated "blastn" at the NCBI web site), using the following parameters: gap penalty = 5; gap extension penalty = 2; mismatch penalty = 3; match reward = 1; expectation value 10.0; and word size = 11 to obtain nucleotide sequences homologous to a nucleic acid described herein. BLAST protein searches can be performed with the XBLAST program (designated "blastn" at the NCBI web site) or the NCBI "blastp" program, using the following parameters: expectation value 10.0, BLOSUM62 scoring matrix to obtain amino acid sequences homologous to a protein molecule described herein.

To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997, Nucleic Acids Res. 25:3389-3402). Alternatively, PSI-Blast or PHI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*id.*) and relationships between molecules which share a common pattern. When utilizing BLAST, Gapped BLAST, PSI-Blast, and PHI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* http://www.ncbi.nlm.nih.gov.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide of the invention. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding proteins of the invention from other species (homologs), which have a nucleotide sequence which differs from that of the mouse proteins described herein are within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and homologs of a cDNA of the invention can be isolated based on their identity to mouse nucleic acid molecules using the mouse cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. For example, a homolog of a nucleic acid encoding a rat REMODELIN protein of the invention can be isolated based on its hybridization with a nucleic acid molecule encoding all or part of rat and/or human REMODELIN under high stringency conditions.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the nucleic acid, peptide, and/or composition of the invention in the kit for effecting alleviation of the various diseases or disorders recited herein. Optionally, or alternately, the instructional material may describe one or more methods of alleviation the diseases or disorders in a cell or a tissue of a mammal. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the nucleic acid, peptide, and/or composition of the invention or be shipped together with a container which contains the nucleic acid, peptide, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, *e.g.*, RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytidine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

As used herein, the term "negative remodeling" (also known as inward remodeling) means a physiologic or pathologic response of a blood vessel to a stimulus

resulting in a reduction of vessel diameter and lumen diameter. Such a stimulus could be provided by, for example, but not limited to, a change in blood flow or an angioplasty procedure.

"Neointima formation," as that term is used herein, means the thickening and enlargement of the tunica intima of a blood vessel due to accumulation of cells and extracellular matrix in this layer of the vessel.

By describing two polynucleotides as "operably linked" is meant that a single-stranded or double-stranded nucleic acid moiety comprises the two polynucleotides arranged within the nucleic acid moiety in such a manner that at least one of the two polynucleotides is able to exert a physiological effect by which it is characterized upon the other. By way of example, a promoter operably linked to the coding region of a gene is able to promote transcription of the coding region.

Preferably, when the nucleic acid encoding the desired protein further comprises a promoter/regulatory sequence, the promoter/regulatory is positioned at the 5' end of the desired protein coding sequence such that it drives expression of the desired protein in a cell. Together, the nucleic acid encoding the desired protein and its promoter/regulatory sequence comprise a "transgene."

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living human cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the

gene product to be produced in a living human cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living human cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

A "polyadenylation sequence" is a polynucleotide sequence which directs the addition of a poly A tail onto a transcribed messenger RNA sequence.

A "polynucleotide" means a single strand or parallel and anti-parallel strands of a nucleic acid. Thus, a polynucleotide may be either a single-stranded or a double-stranded nucleic acid.

The term "nucleic acid" typically refers to large polynucleotides.

The term "oligonucleotide" typically refers to short polynucleotides, generally, no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), this also includes an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T."

Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction.

The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand which are located 5' to a reference point on the DNA are referred to as "upstream sequences"; sequences on the DNA strand which are 3' to a reference point on the DNA are referred to as "downstream sequences."

A "portion" of a polynucleotide means at least at least about twenty sequential nucleotide residues of the polynucleotide. It is understood that a portion of a polynucleotide may include every nucleotide residue of the polynucleotide.

"Primer" refers to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation

for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, *i.e.*, in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be labeled with, *e.g.*, chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

"Probe" refers to a polynucleotide that is capable of specifically hybridizing to a designated sequence of another polynucleotide. A probe specifically hybridizes to a target complementary polynucleotide, but need not reflect the exact complementary sequence of the template. In such a case, specific hybridization of the probe to the target depends on the stringency of the hybridization conditions. Probes can be labeled with, *e.g.*, chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell.

A recombinant polynucleotide may serve a non-coding function (*e.g.*, promoter, origin of replication, ribosome-binding site, etc.) as well.

A "recombinant polypeptide" is one which is produced upon expression of a recombinant polynucleotide.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer.

The term "protein" typically refers to large polypeptides.

The term "peptide" typically refers to short polypeptides.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

As used herein, the term "reporter gene" means a gene, the expression of which can be detected using a known method. By way of example, the *Escherichia coli lacZ* gene may be used as a reporter gene in a medium because expression of the lacZ gene can be detected using known methods by adding the chromogenic substrate *o*-nitrophenyl-*β*-galactoside to the medium (Gerhardt et al., eds., 1994, Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC, p. 574).

As used herein, the term "REMODELIN" means any adventitia-induced and bone expressed molecule having significant sequence identity with REMODELIN disclosed herein. More specifically, the putative REMODELIN will share at least about 33% sequence identity with at least one of a nucleic acid having the sequence SEQ ID NO:1 and a nucleic acid having the sequence SEQ ID NO:3. More preferably, the nucleic acid encoding REMODELIN has at least about 35% identity, even more preferably, at least about 40% identity, yet more preferably, at least about 45% identity, even more preferably, at least about 50% identity, more preferably, at least about 55% identity, even more preferably, at least about 60% identity, yet more preferably, at least about 65% identity, more preferably, at least about 70% identity, yet more preferably, at least about 75% identity, even more preferably, at least about 80% identity, more preferably, at least about 85% identity, yet more preferably, about 90% identity, even more preferably, at least about 95% identity, and most preferably, at least about 99% sequence identity with at least one of SEQ ID NO:1 and SEQ ID NO:3 disclosed herein. Even more preferably, the nucleic acid is at least one of SEQ ID NO:1 and SEQ ID NO:3. Further, the biological activity of a REMODELIN preferably includes inhibition of expression of the nucleic acid encoding the REMODELIN protein by a soluble TGF-β receptor type II (TGF-βRII), which blocks TGF-β signaling. Further, preferably, the biological activity of a REMODELIN molecule includes induction of

expression of the nucleic acid by TGF, induction of expression of the nucleic acid encoding a REMODELIN in a blood vessel following vessel injury, induction of expression of the nucleic acid encoding the protein in fibroblasts during wound healing, expression in osteoblasts during bone formation, causing cell death in endothelial cells when it is overexpressed, involvement in cell-cell and cell-matrix interaction, and affecting cell viability such as by, for example, affecting the life span of a cell.

Further, the data disclosed elsewhere herein demonstrate that REMODELIN plays an important role in bone growth. In one embodiment, transgenic mice over-expressing REMODELIN, similar to data obtained using frog embryos which exhibited failure of dorsal closure, exhibited *spina bifida*-like effects. Therefore, the term "REMODELIN" encompasses a nucleic acid that, when over-expressed in a mammalian embryo, mediates or is associated with altered bone growth, bone density, and/or *spina bifida*-like phenotype.

Unless otherwise indicated, "REMODELIN" encompasses all known REMODELINs (*e.g.*, rat REMODELIN$_S$, rat REMODELIN$_L$, and human REMODELIN), and REMODELINs to be discovered, including but not limited to, mouse REMODELIN, having the characteristics and/or physical features of the REMODELIN disclosed herein.

However, the present invention does not include the isolated nucleic acids having the sequences designated by the following GenBank Accession Numbers: AA335862 (sharing about 87% identity with REMODELIN over about 373 nucleotides); C01758 (sharing about 87% identity with REMODELIN over about 356 nucleotides); AA335551 (sharing about 87% identity with REMODELIN over about 334 nucleotides); and AA406425 (sharing about 88% identity with REMODELIN over about 312 nucleotides); R46857; AA584310; D79314; AI085616; D62262; AA482398; AA482544; AI359844; AI352209; AI239604; AI218433; AI081084; AI074870; AI074769; AA974239; AA969841; AA857920; AA723450; AA410434; AA738416; AI370649; AA507081.

"REMODELIN expression-inhibiting amount," as used herein, means any amount of a substance or molecule that detectably decreases the level of

REMODELIN expression, amount, and/or activity compared with the level of REMODELIN expression, amount, and/or activity in the absence of the substance or molecule. Thus, any amount that mediates a detectable decrease in: the amount of REMODELIN present, the level of REMODELIN mRNA expression, and/or the ability of REMODELIN to form necessary ligand/receptor interactions, is encompassed in the present invention. The assays by which these conditions are examined are well-known in the art and several are exemplified herein.

"REMODELIN expression-increasing amount," as used herein, means any amount of a substance or molecule that detectably increases the level of REMODELIN expression, amount, and/or activity compared with the level of REMODELIN expression, amount, and/or activity in the absence of the substance or molecule. Thus, any amount that mediates a detectable increase in: the amount of REMODELIN present, the level of REMODELIN mRNA expression, and/or the ability of REMODELIN to form necessary ligand/receptor interactions, is encompassed in the present invention. The assays by which these conditions are examined are well-known in the art and several are exemplified herein.

By the term "REMODELIN-like activity," as used herein, refers to the ability of a molecule or compound to be induced by TGF-β, selectively induced in adventitia of injured vessels, to cause phenotypic abnormalities in amphibian embryos such as those disclosed herein (*e.g.*, split tail, abnormal head development, lack of mesoderm development upon FGF-induction, failure of dorsal closure, and the like), to exhibit increased expression only in injured vessel adventitia but not in uninjured vessels nor in the neointima of injured or uninjured vessels, the ability to induce adventitial cell proliferation, to be inhibited by a soluble TGF-β receptor II (which blocks TGF-β signaling), the ability to be induced in fibroblasts during wound healing, the ability to be expressed by osteoblasts during bone formation, the ability to be expressed in osteoblasts adjacent to mineralized bone, the ability to be strongly expressed along full thickness skin incisions undergoing wound healing and remodeling, the ability to mediate cell death in endothelial cells when overexpressed, the ability to inhibit cell adhesion and cell-cell interaction when an antisense nucleic

complementary to the nucleic acid encoding the molecule is expressed in a cell, and the ability to mediate excessive or insufficient wound healing responses, scarring, keloids, bone formation, bone density, lack of dorsal closure, *spina bifida*-like effects, fracture healing, and the like.

A "restriction site" is a portion of a double-stranded nucleic acid which is recognized by a restriction endonuclease.

A portion of a double-stranded nucleic acid is "recognized" by a restriction endonuclease if the endonuclease is capable of cleaving both strands of the nucleic acid at the portion when the nucleic acid and the endonuclease are contacted.

By the term "specifically binds," as used herein, is meant a compound, *e.g.*, a protein, a nucleic acid, an antibody, and the like, which recognizes and binds a specific molecule, but does not substantially recognize or bind other molecules in a sample.

A first oligonucleotide anneals with a second oligonucleotide "with high stringency" if the two oligonucleotides anneal under conditions whereby only oligonucleotides which are at least about 73%, more preferably, at least about 75%, even more preferably, at least about 80%, even more preferably, at least about 85%, yet more preferably, at least about 90%, and most preferably, at least about 95%, complementary anneal with one another. The stringency of conditions used to anneal two oligonucleotides is a function of, among other factors, temperature, ionic strength of the annealing medium, the incubation period, the length of the oligonucleotides, the G-C content of the oligonucleotides, and the expected degree of non-homology between the two oligonucleotides, if known. Methods of adjusting the stringency of annealing conditions are known (*see, e.g.*, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

As used herein, the term "transgene" means an exogenous nucleic acid sequence which exogenous nucleic acid is encoded by a transgenic cell or mammal.

A "recombinant cell" is a cell that comprises a transgene. Such a cell may be a eukaryotic cell or a prokaryotic cell. Also, the transgenic cell encompasses, but is not limited to, an embryonic stem cell comprising the transgene, a cell obtained from a chimeric mammal derived from a transgenic ES cell where the cell comprises

the transgene, a cell obtained from a transgenic mammal, or fetal or placental tissue thereof, and a prokaryotic cell comprising the transgene.

By the term "exogenous nucleic acid" is meant that the nucleic acid has been introduced into a cell or an animal using technology which has been developed for the purpose of facilitating the introduction of a nucleic acid into a cell or an animal.

By "tag" polypeptide is meant any protein which, when linked by a peptide bond to a protein of interest, may be used to localize the protein, to purify it from a cell extract, to immobilize it for use in binding assays, or to otherwise study its biological properties and/or function.

"TGF-β signaling", as the term is used herein is any signal mediated by TGF-β interaction with a cognate receptor, *e.g.*, TGF-β receptor type II. One result of such interaction is an increase in REMODELIN receptor in a cell comprising the receptor on its surface. Other effects of TGF-β signaling are well-known in the art and can be assessed using standard methods also known in the relevant art.

As used herein, the term "transgenic mammal" means a mammal, the germ cells of which comprise an exogenous nucleic acid.

As used herein, to "treat" means reducing the frequency with which symptoms of arterial restenosis, adventitial fibrosis, excessive or insufficient wound healing responses, scarring, keloids, bone formation, fracture healing, and the like, are experienced by a patient.

By the term "vector" as used herein, is meant any plasmid or virus encoding an exogenous nucleic acid. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into virions or cells, such as, for example, polylysine compounds and the like. The vector may be a viral vector which is suitable as a delivery vehicle for delivery of the REMODELIN protein or nucleic acid encoding a mammalian REMODELIN, to the patient, or the vector may be a non-viral vector which is suitable for the same purpose.

Examples of viral and non-viral vectors for delivery of DNA to cells and tissues are well known in the art and are described, for example, in Ma et al. (1997, Proc. Natl. Acad. Sci. U.S.A. 94:12744-12746). Examples of viral vectors include, but are not limited to, a recombinant vaccinia virus, a recombinant adenovirus, a

recombinant retrovirus, a recombinant adeno-associated virus, a recombinant avian pox virus, and the like (Cranage et al., 1986, EMBO J. 5:3057-3063; International Patent Application No. WO94/17810, published August 18, 1994; International Patent Application No. WO94/23744, published October 27, 1994). Examples of non-viral vectors include, but are not limited to, liposomes, polyamine derivatives of DNA, and the like.

A "knock-out targeting vector," as the term is used herein, means a vector comprising two nucleic acid sequences each of which is complementary to a nucleic acid regions flanking a target sequence of interest which is to be deleted and/or replaced by another nucleic acid sequence. The two nucleic acid sequences therefore flank the target sequence which is to be removed by the process of homologous recombination

Description

I. Isolated nucleic acids

A. Sense nucleic acids

The present invention includes an isolated nucleic acid encoding a mammalian adventitia-inducible and bone expressed molecule, REMODELIN, or a fragment thereof, wherein the nucleic acid shares at least about 33% identity with at least one nucleic acid having the sequence of (SEQ ID NO:1) and (SEQ ID NO:3). Preferably, the nucleic acid is at least about 35% homologous, more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to at least one of

SEQ ID NO:1 and SEQ ID NO:3 disclosed herein. Even more preferably, the nucleic acid is at least one of SEQ ID NO:1 and SEQ ID NO:3.

The present invention includes an isolated nucleic acid encoding rat REMODELIN, or a fragment thereof, wherein the nucleic acid shares at least about 33% homology with a nucleic acid having the sequence SEQ ID NO:1. Preferably, the nucleic acid is at least about 35% homologous, more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to the rat REMODELIN disclosed herein (SEQ ID NO:1). Even more preferably, the nucleic acid is SEQ ID NO:1.

The present invention includes an isolated nucleic acid encoding human REMODELIN, or a fragment thereof, wherein the nucleic acid shares at least about 33% homology with human REMODELIN (SEQ ID NO:3). Preferably, the nucleic acid is at least about 35% homologous, more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to the human REMODELIN disclosed herein (SEQ ID NO:3). Even more preferably, the nucleic acid is SEQ ID NO:3.

In another aspect, the present invention includes an isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof, wherein the protein encoded by the nucleic acid shares greater than about 6% homology with the amino acid sequence of at least one of SEQ ID NO:2 (rat REMODELIN$_S$), SEQ ID NO:4 (human REMODELIN), and SEQ ID NO:5 (rat REMODELIN$_L$). That is, searching GenBank databases disclosed that REMODELIN shares about 62% sequence identity with a portion of the sequence GenBank Acc. No. P27393, collagen alpha-2 (IV) chain precursor, over a stretch of about 35 amino acids. Full-length REMODELIN protein comprises about 243 amino acids such that full-length REMODELIN shares about 5.7% overall sequence identity with collagen alpha-2 (IV) chain precursor (*i.e.*, GenBank Acc. No. P27393).

Preferably, the protein encoded by the isolated nucleic acid encoding REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5. Even more preferably, the REMODELIN protein encoded by the nucleic acid is at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

In another aspect, the present invention includes an isolated nucleic acid encoding rat REMODELIN, or a fragment thereof, wherein the protein encoded by the nucleic acid shares at least about 6% homology with the amino acid sequence of SEQ ID NO:2. Preferably, the protein encoded by the isolated nucleic acid encoding

REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to rat REMODELIN disclosed herein (SEQ ID NO:2). Even more preferably, the rat REMODELIN protein encoded by the nucleic acid is SEQ ID NO:2.

In another aspect, the present invention includes an isolated nucleic acid encoding human REMODELIN, or a fragment thereof, wherein the protein encoded by the nucleic acid shares at least about 6% homology with the amino acid sequence of SEQ ID NO:4. Preferably, the protein encoded by the isolated nucleic acid encoding REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to the human

REMODELIN disclosed herein (SEQ ID NO:4). Even more preferably, the human REMODELIN protein encoded by the nucleic acid is SEQ ID NO:4.

One skilled in the art would understand, based upon the disclosure provided herein, that a nucleic acid encoding a rat REMODELIN (SEQ ID NO:1) can be alternatively translated to produce an alternate rat REMODELIN protein comprising 245 amino acids (rat REMODELIN$_S$; SEQ ID NO:2) and a protein comprising an additional 32 amino acid residues at the N-terminus (*i.e.*, the 277 amino acid long form of REMODELIN designated REMODELIN$_L$ [SEQ ID NO:5]) since the nucleic acid encoding rat REMODELIN (SEQ ID NO:1) comprises two putative transcriptional start sites at positions 19 and 116 (Figure 4A) that are compatible with the Kozak rule.

Therefore, in another aspect, the present invention includes an isolated nucleic acid encoding rat REMODELIN, or a fragment thereof, wherein the protein encoded by the nucleic acid shares at least about 6% homology with the amino acid sequence of SEQ ID NO:5 (*i.e.*, 277 amino acid rat REMODELIN$_L$). Preferably, the protein encoded by the isolated nucleic acid encoding REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to the rat REMODELIN$_L$ disclosed herein (SEQ ID NO:5). Even more preferably, the rat REMODELIN$_L$ protein encoded by the nucleic acid is SEQ ID NO:5.

One skilled in the art would appreciate, based upon the disclosure provided herein, that a mouse REMODELIN homolog likely exists and can be readily

identified and isolated using the methods described herein using the sequence data disclosed herein regarding the highly-conserved rat and mouse homologs. Thus, the present invention encompasses additional REMODELINs that can be readily identified based upon the disclosure provided herein, including, but not limited to, mouse REMODELIN.

The isolated nucleic acid of the invention should be construed to include an RNA or a DNA sequence encoding a REMODELIN protein of the invention (excluding the isolated nucleic acids referred to by the following GenBank Accession Nos: AA335862; C01758; AA335551; AA406425; R46857; AA584310; D79314; AI085616; D62262; AA482398; AA482544; AI359844; AI352209; AI239604; AI218433; AI081084; AI074870; AI074769; AA974239; AA969841; AA857920; AA723450; AA410434; AA738416; AI370649; AA507081), and any modified forms thereof, including chemical modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Chemical modifications of nucleotides may also be used to enhance the efficiency with which a nucleotide sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

The present invention should not be construed as being limited solely to the nucleic and amino acid sequences disclosed herein. Once armed with the present invention, it is readily apparent to one skilled in the art that other nucleic acids encoding REMODELIN proteins can such as those present in other species of mammals (*e.g.*, ape, gibbon, bovine, ovine, equine, porcine, canine, feline, and the like) be obtained by following the procedures described herein in the experimental details section for the isolation of the rat, and human REMODELIN nucleic acids encoding REMODELIN polypeptides as disclosed herein (*e.g.*, screening of genomic or cDNA libraries), and procedures that are well-known in the art (*e.g.*, reverse transcription PCR using mRNA samples) or to be developed.

Further, any number of procedures may be used for the generation of mutant, derivative or variant forms of REMODELIN using recombinant DNA methodology well known in the art such as, for example, that described in Sambrook et

al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York).

Procedures for the introduction of amino acid changes in a protein or polypeptide by altering the DNA sequence encoding the polypeptide are well known in the art and are also described in Sambrook et al. (1989, *supra*); Ausubel et al. (1997, *supra*).

The invention includes a nucleic acid encoding a mammalian REMODELIN wherein a nucleic acid encoding a tag polypeptide is covalently linked thereto. That is, the invention encompasses a chimeric nucleic acid wherein the nucleic acid sequences encoding a tag polypeptide is covalently linked to the nucleic acid encoding at least one of rat REMODELIN and human REMODELIN. Such tag polypeptides are well known in the art and include, for instance, green fluorescent protein (GFP), an influenza virus hemagglutinin tag polypeptide, myc, myc-pyruvate kinase (myc-PK), His_6, maltose biding protein (MBP), a FLAG tag polypeptide, and a glutathione-S-transferase (GST) tag polypeptide. However, the invention should in no way be construed to be limited to the nucleic acids encoding the above-listed tag polypeptides. Rather, any nucleic acid sequence encoding a polypeptide which may function in a manner substantially similar to these tag polypeptides should be construed to be included in the present invention.

The nucleic acid comprising a nucleic acid encoding a tag polypeptide can be used to localize REMODELIN within a cell, a tissue (*e.g.*, a blood vessel, bone, and the like), and/or a whole organism (*e.g.*, an amphibian and/or a mammalian embryo, and the like), detect REMODELIN if secreted from a cell, and to study the role(s) of REMODELIN in a cell. Further, addition of a tag polypeptide facilitates isolation and purification of the "tagged" protein such that the proteins of the invention can be produced and purified readily.

B. Antisense nucleic acids

In certain situations, it may be desirable to inhibit expression of REMODELIN and the invention therefore includes compositions useful for inhibition

of REMODELIN expression. Thus, the invention features an isolated nucleic acid complementary to a portion or all of a nucleic acid encoding a mammalian REMODELIN, which nucleic acid is in an antisense orientation with respect to transcription. Preferably, the antisense nucleic acid is complementary with a nucleic acid having at least about 33% homology with at least one of SEQ ID NO:1 and SEQ ID NO:3, or a fragment thereof. Preferably, the nucleic acid is at least about 35% homologous, more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to a nucleic acid complementary to a portion or all of a nucleic acid encoding a mammalian REMODELIN having the sequence of at least one of SEQ ID NO:1 and SEQ ID NO:3, or a fragment thereof, which is in an antisense orientation with respect to transcription. Most preferably, the nucleic acid is complementary to a portion or all of a nucleic acid that is at least one of SEQ ID NO:1 and SEQ ID NO:3, or a fragment thereof. Such antisense nucleic acid serves to inhibit the expression, function, or both, of an adventitia-inducible and bone expressed (REMODELIN) molecule.

In one aspect, the invention includes an isolated nucleic acid complementary to a portion or all of a nucleic acid encoding a mammalian REMODELIN molecule, which nucleic acid is in an antisense orientation with respect to transcription. Preferably, the antisense nucleic acid is complementary with a nucleic acid having at least about 33% homology with SEQ ID NO:1, or a fragment thereof. Preferably, the antisense nucleic acid is complementary with a nucleic acid having at least about 35% identity, more preferably, at least about 40% identity, even more preferably, at least about 45% identity, yet more preferably, at least about 50% identity, more preferably, at least about 55% identity, more preferably, at least about 60%

identity, even more preferably, at least about 65% identity, yet more preferably, at least about 70% identity, more preferably, at least about 75% identity, even more preferably, at least about 80% identity, yet more preferably, at least about 85% identity, more preferably, at least about 90% identity, even more preferably, at least about 95% identity, and most preferably, at least about 99% identity with a nucleic acid complementary to a portion or all of a nucleic acid encoding a mammalian REMODELIN having the sequence SEQ ID NO:1

Most preferably, the nucleic acid is complementary to a portion or all of a nucleic acid that is SEQ ID NO:1, or a fragment thereof. Such antisense nucleic acid serves to inhibit the expression, function, or both, of an adventitia-inducible and bone expressed REMODELIN molecule.

In another aspect, the invention includes an isolated nucleic acid complementary to a portion or all of a nucleic acid encoding a mammalian REMODELIN molecule, which nucleic acid is in an antisense orientation with respect to transcription. Preferably, the antisense nucleic acid is complementary with a nucleic acid having at least about 35% identity, more preferably, at least about 40% identity, even more preferably, at least about 45% identity, yet more preferably, at least about 50% identity, more preferably, at least about 55% identity, more preferably, at least about 60% identity, even more preferably, at least about 65% identity, yet more preferably, at least about 70% identity, more preferably, at least about 75% identity, even more preferably, at least about 80% identity, yet more preferably, at least about 85% identity, more preferably, at least about 90% identity, even more preferably, at least about 95% identity, and most preferably, at least about 99% identity with a nucleic acid complementary to a nucleic acid encoding a mammalian REMODELIN having the sequence SEQ ID NO:3, or a fragment thereof. Most preferably, the nucleic acid is complementary to a portion or all of a nucleic acid that is SEQ ID NO:3, or a fragment thereof. Such antisense nucleic acid serves to inhibit the expression, function, or both, of an adventitia-inducible and bone expressed REMODELIN molecule.

Further, antisense nucleic acids complementary to all or a portion of a nucleic acid encoding REMODELIN can be used to detect the expression of

REMODELIN mRNA in a cell, tissue, and/or organism, using, for example but not limited to, *in situ* hybridization. Thus, one skilled in the art would understand, based upon the disclosure provided herein, that the invention encompasses antisense nucleic acids that can be used as probes to assess REMODELIN expression. Such antisense nucleic acids encompass, but are not limited to, a nucleic acid having the sequence SEQ ID NO:6.

Antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of between about 10 to about 30, and more preferably about 15 nucleotides, are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated by the invention include oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* Cohen, *supra*; Tullis, 1991, U.S. Patent No. 5,023,243, incorporated by reference herein in its entirety).

II. Isolated polypeptides

The invention also includes an isolated polypeptide comprising a mammalian REMODELIN. Preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least about 6% homologous to a polypeptide having the amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5. Preferably, the isolated polypeptide is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous

to at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5. More preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least one of rat REMODELIN$_S$, human REMODELIN, and rat REMODELIN$_L$. Most preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

The invention also includes an isolated polypeptide comprising a mammalian REMODELIN molecule. Preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least about 6% homologous to a polypeptide having the amino acid sequence of SEQ ID NO:2. More preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to rat REMODELIN$_S$ (SEQ ID NO:2) More preferably, the isolated polypeptide comprising a mammalian REMODELIN is rat REMODELIN$_S$. Most preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is SEQ ID NO:2.

The invention also includes an isolated polypeptide comprising a mammalian REMODELIN molecule. Preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is at least about 6% homologous to a polypeptide having the amino acid sequence of SEQ ID NO:4. More preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably,

at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to SEQ ID NO:4. More preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is human REMODELIN. Most preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is SEQ ID NO:4.

The invention also includes an isolated polypeptide comprising a mammalian REMODELIN molecule. Preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is at least about 6% homologous to a polypeptide having the amino acid sequence of SEQ ID NO:5. More preferably, the isolated polypeptide comprising a mammalian REMODELIN is at least about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to rat REMODELIN$_L$. More preferably, the

isolated polypeptide comprising a mammalian REMODELIN molecule is rat REMODELIN$_L$. Most preferably, the isolated polypeptide comprising a mammalian REMODELIN molecule is SEQ ID NO:5.

The present invention also provides for analogs of proteins or peptides which comprise a REMODELIN as disclosed herein. Analogs may differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both. For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro*, chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-

naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

The present invention should also be construed to encompass "mutants," "derivatives," and "variants" of the peptides of the invention (or of the DNA encoding the same) which mutants, derivatives and variants are REMODELIN peptides which are altered in one or more amino acids (or, when referring to the nucleotide sequence encoding the same, are altered in one or more base pairs) such that the resulting peptide (or DNA) is not identical to the sequences recited herein, but has the same biological property as the peptides disclosed herein, in that the peptide has biological/biochemical properties of the REMODELIN peptide of the present invention.

A "biological property of a REMODELIN protein' should be construed but not be limited to include, the ability of the expression of the peptide to be induced by TGF-β, the ability of the peptide to be expressed selectively in adventitia, the ability of the peptide to be induced by balloon-injury, the ability of the peptide to be expressed in bone, the ability of the peptide to be expressed in a mouse embryo commencing at about day 11.5 post coitus, the ability of a molecule to be selectively induced in adventitia of injured vessels, to cause phenotypic abnormalities in amphibian embryos such as those disclosed herein (*e.g.*, split tail, abnormal head development, lack of mesoderm development upon FGF-induction, failure of dorsal closure, and the like), to exhibit increased expression only in injured vessel adventitia but not in uninjured vessels nor in the neointima of injured or uninjured vessels, the ability to induce adventitial cell proliferation, to be inhibited by a soluble TGF-β receptor II (which blocks TGF-β signaling), the ability to be induced in fibroblasts during wound healing, the ability to be expressed by osteoblasts during bone formation, the ability to mediate cell death in endothelial cells when overexpressed, the ability to affect cell adhesion and cell-cell interaction, the ability to affect bone density and/or bone growth, and the ability to mediate excessive or insufficient wound healing responses, scarring, keloids, bone formation, fracture healing, and the like.

Further, the invention should be construed to include naturally occurring variants or recombinantly derived mutants of REMODELIN sequences, which variants

or mutants render the protein encoded thereby either more, less, or just as biologically active as the full-length clones of the invention.

The nucleic acids, and peptides encoded thereby, are useful tools for elucidating the function(s) of REMODELIN molecule in a cell. Further, nucleic and amino acids comprising mammalian REMODELIN molecule are useful diagnostics which can be used, for example, to identify a compound that affects REMODELIN expression and/or TGF-β signaling, and the like, and is a potential therapeutic drug candidate for arterial restenosis, anti-cancer therapy, to promote or inhibit wound healing, to inhibit scar tissue or keloid formation, to promote bone fracture healing, and the like. The nucleic acids, the proteins encoded thereby, or both, can be administered to a mammal to increase or decrease expression of REMODELIN in the mammal. This can be beneficial for the mammal in situations where under or over-expression of REMODELIN in the mammal mediates a disease or condition associated with altered expression of REMODELIN compared with normal expression of REMODELIN in a healthy mammal. Such conditions that can be affected by modulating REMODELIN expression thereby providing a therapeutic benefit include, but are not limited to, wound healing, arterial injury, ossification, and the like. This is because, as more fully disclosed elsewhere herein, REMODELIN is transiently expressed in (myo)fibroblasts in conditions associated with healing and repair following tissue injury. For instance, REMODELIN is expressed in osteoblasts bone, which is undergoing constant remodeling. Additionally, over-expression of REMODELIN during embryogenesis affects dorsal closure, bone density, and bone growth, and mediates and/or is associated with *spina bifida*-like effects all of which demonstrate the important biological role(s) of REMODELIN.

Additionally, the nucleic and amino acids of the invention can be used to produce recombinant cells and transgenic non-human mammals which are useful tools for the study of REMODELIN action, the identification of novel diagnostics and therapeutics for treatment, and for elucidating the cellular role(s) of REMODELIN, among other things. For instance, transgenic animals can be used to study bone related, wound healing related, and vascular disease related conditions.

Further, the nucleic and amino acids of the invention can be used diagnostically, either by assessing the level of gene expression or protein expression, to assess severity and prognosis of negative remodeling, arterial restenosis, vessel injury, fibrosis, bone growth, and the like. The nucleic acids and proteins of the invention are also useful in the development of assays to assess the efficacy of a treatment for preventing arterial restenosis, affecting bone density and bone growth, and the like. That is, the nucleic acids and polypeptides of the invention can be used to detect the effect of various therapies on REMODELIN expression, thereby ascertaining the effectiveness of the therapies such as, but not limited to, assessment of treatment efficacies for restenosis, anti-fibrotic therapy in any tissue, therapies to promote wound healing in any tissue and therapies for bone formation including bone fracture healing.

III. Vectors

In other related aspects, the invention includes an isolated nucleic acid encoding a mammalian REMODELIN operably linked to a nucleic acid comprising a promoter/regulatory sequence such that the nucleic acid is preferably capable of directing expression of the protein encoded by the nucleic acid. Thus, the invention encompasses expression vectors and methods for the introduction of exogenous DNA into cells with concomitant expression of the exogenous DNA in the cells such as those described, for example, in Sambrook et al. (1989, *supra*), and Ausubel et al. (1997, *supra*).

Expression of REMODELIN, either alone or fused to a detectable tag polypeptide, in cells which either do not normally express the REMODELIN or which do not express REMODELIN fused with a tag polypeptide, may be accomplished by generating a plasmid, viral, or other type of vector comprising the desired nucleic acid operably linked to a promoter/regulatory sequence which serves to drive expression of the protein, with or without tag, in cells in which the vector is introduced. Many promoter/regulatory sequences useful for driving constitutive expression of a gene are available in the art and include, but are not limited to, for example, the cytomegalovirus immediate early promoter enhancer sequence, the SV40 early promoter, both of which were used in the experiments disclosed herein, as well as the

Rous sarcoma virus promoter, and the like. Moreover, inducible and tissue specific expression of the nucleic acid encoding REMODELIN may be accomplished by placing the nucleic acid encoding REMODELIN, with or without a tag, under the control of an inducible or tissue specific promoter/regulatory sequence. Examples of tissue specific or inducible promoter/regulatory sequences which are useful for his purpose include, but are not limited to the MMTV LTR inducible promoter, and the SV40 late enhancer/promoter. In addition, promoters which are well known in the art which are induced in response to inducing agents such as metals, glucocorticoids, and the like, are also contemplated in the invention. Thus, it will be appreciated that the invention includes the use of any promoter/regulatory sequence, which is either known or unknown, and which is capable of driving expression of the desired protein operably linked thereto.

Expressing REMODELIN using a vector allows the isolation of large amounts of recombinantly produced protein. Further, where the lack or decreased level of REMODELIN expression causes a disease, disorder, or condition associated with such expression, the expression of REMODELIN driven by a promoter/regulatory sequence can provide useful therapeutics including, but not limited to, gene therapy whereby REMODELIN is provided. A disease, disorder or condition associated with a decreased level of expression, level of protein, or decreased activity of the protein, for which administration of REMODELIN can be useful can include, but is not limited to, bone formation, bone fracture healing, wound healing and repair in any tissue, and the like. Therefore, the invention includes not only methods of inhibiting REMODELIN expression, translation, and/or activity, but it also includes methods relating to increasing REMODELIN expression, protein level, and/or activity since both decreasing and increasing REMODELIN expression and/or activity can be useful in providing effective therapeutics.

One skilled in the art would appreciate, based upon the disclosure provided herein, that because of the selective expression of REMODELIN during wound healing in response to injury in any tissue, the promoter for REMODELIN can be an excellent choice for targeting nucleic acid expression of a desired gene to a site of tissue injury.

Selection of any particular plasmid vector or other DNA vector is not a limiting factor in this invention and a wide plethora vectors is well-known in the art. Further, it is well within the skill of the artisan to choose particular promoter/regulatory sequences and operably link those promoter/regulatory sequences to a DNA sequence encoding a desired polypeptide. Such technology is well known in the art and is described, for example, in Sambrook, *supra*, and Ausubel, *supra*.

The invention thus includes a vector comprising an isolated nucleic acid encoding a mammalian REMODELIN. The incorporation of a desired nucleic acid into a vector and the choice of vectors is well-known in the art as described in, for example, Sambrook et al., *supra*, and Ausubel et al., *supra*.

The invention also includes cells, viruses, proviruses, and the like, containing such vectors. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. *See, e.g.*, Sambrook et al., *supra*; Ausubel et al., *supra*.

The nucleic acids encoding REMODELIN can be cloned into various plasmid vectors. However, the present invention should not be construed to be limited to plasmids or to any particular vector. Instead, the present invention should be construed to encompass a wide plethora of vectors which are readily available and/or well-known in the art and no vector at all.

IV. Antisense molecules and ribozymes

Further, the invention includes a recombinant cell comprising an antisense nucleic acid which cell is a useful model for elucidating the role(s) of REMODELIN in cellular processes. That is, the increased expression of REMODELIN in balloon-injured vessels and, more specifically, in the adventitia thereof, indicate that REMODELIN is involved in cell proliferation associated with negative remodeling and arterial restenosis. Accordingly, a transgenic cell comprising an antisense nucleic acid complementary to REMODELIN but in an antisense orientation is a useful tool for the study of the mechanism(s) of action of

REMODELIN and its role(s) in the cell and for the identification of therapeutics that ameliorate the effect(s) of REMODELIN expression.

One skilled in the art can appreciate, based upon the disclosure provided herein, that an antisense nucleic acid complementary to a nucleic acid encoding REMODELIN can be used to transfect a cell and the cell can be studied to determine the effect(s) of altered expression of REMODELIN in order to study the function(s) of REMODELIN and to identity useful therapeutics and diagnostics.

Further, methods of decreasing REMODELIN expression and/or activity in a cell can provide useful diagnostics and/or therapeutics for diseases, disorders or conditions mediated by or associated with increased REMODELIN expression, increased level of REMODELIN protein in a cell or secretion therefrom, and/or increased REMODELIN activity. Such diseases, disorders or conditions include, but are not limited to, any condition associated with fibrosis, *e.g.*, proliferation of fibroblasts with or without excessive fibrous tissue formation, and any condition associated with excessive bone formation or ectopic ossification (malignant or benign), and the like.

One skilled in the art will appreciate that one way to decrease the levels of REMODELIN mRNA and/or protein in a cell is to inhibit expression of the nucleic acid encoding the protein. Expression of REMODELIN may be inhibited using, for example, antisense molecules, and also by using ribozymes or double-stranded RNA as described in, for example, Wianny and Kernicka-Goetz (2000, Nature Cell Biol. 2:70-75).

Antisense molecules and their use for inhibiting gene expression are well known in the art (*see, e.g.,* Cohen, 1989, In: Oligodeoxyribonucleotides, Antisense Inhibitors of Gene Expression, CRC Press). Antisense nucleic acids are DNA or RNA molecules that are complementary, as that term is defined elsewhere herein, to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American 262:40). In the cell, antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule thereby inhibiting the translation of genes.

The use of antisense methods to inhibit the translation of genes is known in the art, and is described, for example, in Marcus-Sakura (1988, Anal. Biochem. 172:289). Such antisense molecules may be provided to the cell via genetic expression using DNA encoding the antisense molecule as taught by Inoue (1993, U.S. Patent No. 5,190,931).

Alternatively, antisense molecules of the invention may be made synthetically and then provided to the cell. Antisense oligomers of between about 10 to about 30, and more preferably about 15 nucleotides, are preferred, since they are easily synthesized and introduced into a target cell. Synthetic antisense molecules contemplated by the invention include oligonucleotide derivatives known in the art which have improved biological activity compared to unmodified oligonucleotides (*see* Cohen, *supra*; Tullis, 1991, U.S. Patent No. 5,023,243, incorporated by reference herein in its entirety).

Ribozymes and their use for inhibiting gene expression are also well known in the art (*see, e.g.,* Cech et al., 1992, J. Biol. Chem. 267:17479-17482; Hampel et al., 1989, Biochemistry 28:4929-4933; Eckstein et al., International Publication No. WO 92/07065; Altman et al., U.S. Patent No. 5,168,053, incorporated by reference herein in its entirety). Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences encoding these RNAs, molecules can be engineered to recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, J. Amer. Med. Assn. 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes, namely, tetrahymena-type (Hasselhoff, 1988, Nature 334:585) and hammerhead-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating specific mRNA species, and 18-base recognition

sequences are preferable to shorter recognition sequences which may occur randomly within various unrelated mRNA molecules.

Ribozymes useful for inhibiting the expression of REMODELIN can be designed by incorporating target sequences into the basic ribozyme structure which are complementary to the mRNA sequence of the REMODELIN encoded by REMODELIN or having at least about 33% homology to at least one of SEQ ID NO:1 and SEQ ID NO:3. Preferably, the sequence is at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably, at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to at least one of SEQ ID NO:1 and SEQ ID NO:3. Ribozymes targeting REMODELIN may be synthesized using commercially available reagents (Applied Biosystems, Inc., Foster City, CA) or they may be genetically expressed from DNA encoding them.

V. Recombinant cells and transgenic non-human mammals

The invention includes a recombinant cell comprising , *inter alia*, an isolated nucleic acid encoding REMODELIN, an antisense nucleic acid complementary thereto, a nucleic acid encoding an antibody that specifically binds REMODELIN, and the like. In one aspect, the recombinant cell can be transiently transfected with a vector (*e.g.*, a plasmid, and the like) encoding a portion of the nucleic acid encoding REMODELIN. The nucleic acid need not be integrated into the cell genome nor does it need to be expressed in the cell. Moreover, the cell may be a prokaryotic or a eukaryotic cell and the invention should not be construed to be limited to any particular cell line or cell type. Such cells include, but are not limited to, fibroblasts, mouse stem

cells, amphibian oocytes, osteoblasts, smooth muscle cells, endothelial cells, and the like.

In one aspect, the recombinant cell comprising an isolated nucleic acid encoding mammalian REMODELIN is used to produce a transgenic non-human mammal. That is, the exogenous nucleic acid, or "transgene" as it is also referred to herein, of the invention is introduced into a cell, and the cell is then used to generate the non-human transgenic mammal. The cell into which the transgene is introduced is preferably an embryonic stem (ES) cell. However, the invention should not be construed to be limited solely to ES cells comprising the transgene of the invention nor to cells used to produce transgenic animals. Rather, a transgenic cell of the invention includes, but is not limited to, any cell derived from a transgenic animal comprising a transgene, a cell comprising the transgene derived from a chimeric animal derived from the transgenic ES cell, and any other comprising the transgene which may or may not be used to generate a non-human transgenic mammal.

Further, it is important to note that the purpose of transgene-comprising, *i.e.*, recombinant, cells should not be construed to be limited to the generation of transgenic mammals. Rather, the invention should be construed to include any cell type into which a nucleic acid encoding a mammalian REMODELIN is introduced, including, without limitation, a prokaryotic cell and a eukaryotic cell comprising an isolated nucleic acid encoding mammalian REMODELIN.

When the cell is a eukaryotic cell, the cell may be any eukaryotic cell which, when the transgene of the invention is introduced therein, and the protein encoded by the desired gene is no longer expressed therefrom, a benefit is obtained. Such a benefit may include the fact that there has been provided a system in which lack of expression of the desired gene can be studied *in vitro* in the laboratory or in a mammal in which the cell resides, a system wherein cells comprising the introduced gene deletion can be used as research, diagnostic and therapeutic tools, and a system wherein animal models are generated which are useful for the development of new diagnostic and therapeutic tools for selected disease states in a mammal including, for example, negative remodeling, arterial restenosis, and the like. That is, one skilled in the art would appreciate, based upon the disclosure provided herein, that because

proliferation of fibroblasts with scar tissue formation is part of any would healing process, selected disease states or processes associated with such proliferation that can be investigated by assessing REMODELIN expression include, but are not limited to, wound healing, bone formation, bone fracture healing, and fibrosis of any organ.

Alternatively, the invention includes a eukaryotic cell which, when the transgene of the invention is introduced therein, and the protein encoded by the desired gene is expressed therefrom where it was not previously present or expressed in the cell or where it is now expressed at a level or under circumstances different than that before the transgene was introduced, a benefit is obtained. Such a benefit may include the fact that there has been provided a system in the expression of the desired gene can be studied *in vitro* in the laboratory or in a mammal in which the cell resides, a system wherein cells comprising the introduced gene can be used as research, diagnostic and therapeutic tools, and a system wherein animal models are generated which are useful for the development of new diagnostic and therapeutic tools for selected disease states in a mammal.

Such cell expressing an isolated nucleic acid encoding REMODELIN can be used to provide REMODELIN to a cell, tissue, or whole animal where a higher level of REMODELIN can be useful to treat or alleviate a disease, disorder or condition associated with low level of REMODELIN expression and/or activity. Such diseases, disorders or conditions can include, but are not limited to, wound healing, bone formation, and bone fracture healing, and the like. Moreover, one skilled in the art would understand that one goal of a wound-healing response is to regain mechanical strength and structural support. REMODELIN is expressed during such a healing response. Additional expression of REMODELIN could thus lead to accelerated wound healing, bone growth, and fracture healing. Therefore, the invention includes a cell expressing REMODELIN to increase or induce REMODELIN expression, translation, and/or activity, where increasing REMODELIN expression, protein level, and/or activity can be useful to treat or alleviate a disease, disorder or condition.

One of ordinary skill would appreciate, based upon the disclosure provided herein, that a "knock-in" or "knock-out" vector of the invention comprises at least two sequences homologous to two portions of the nucleic acid which is to be

replaced or deleted, respectively. The two sequences are homologous with sequences that flank the gene; that is, one sequence is homologous with a region at or near the 5' portion of the coding sequence of the nucleic acid encoding REMODELIN and the other sequence is further downstream from the first. One skilled in the art would appreciate, based upon the disclosure provided herein, that the present invention is not limited to any specific flanking nucleic acid sequences. Instead, the targeting vector may comprise two sequences which remove some or all (*i.e.*, a "knock-out" vector) or which insert (*i.e.*, a "knock-in" vector) a nucleic acid encoding REMODELIN, or a fragment thereof, from or into a mammalian genome, respectively. The crucial feature of the targeting vector is that it comprise sufficient portions of two sequences located towards opposite, *i.e.*, 5' and 3', ends of the REMODELIN open reading frame (ORF) in the case of a "knock-out" vector, to allow deletion/insertion by homologous recombination to occur such that all or a portion of the nucleic acid encoding REMODELIN is deleted from or inserted into a location on a mammalian chromosome.

The design of transgenes and knock-in and knock-out targeting vectors is well-known in the art and is described in standard treatises such as Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York), and the like. The upstream and downstream portions flanking or within the REMODELIN coding region to be used in the targeting vector may be easily selected based upon known methods and following the teachings disclosed herein based on the disclosure provided herein including the nucleic and amino acid sequences of both rat and human REMODELIN. Armed with these sequences, one of ordinary skill in the art would be able to construct the transgenes and knock-out vectors of the invention.

The invention further includes a knock-out targeting vector comprising a nucleic acid encoding a selectable marker such as, for example, a nucleic acid encoding the *neo*^{R} gene thereby allowing the selection of transgenic a cell where the nucleic acid encoding REMODELIN, or a portion thereof, has been deleted and replaced with the neomycin resistance gene by the cell's ability to grow in the presence

of G418. However, the present invention should not be construed to be limited to neomycin resistance as a selectable marker. Rather, other selectable markers well-known in the art may be used in the knock-out targeting vector to allow selection of recombinant cells where the REMODELIN gene has been deleted and/or inactivated and replaced by the nucleic acid encoding the selectable marker of choice. Methods of selecting and incorporating a selectable marker into a vector are well-known in the art and are describe in, for example, Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

As noted herein, the invention includes a non-human transgenic mammal comprising an exogenous nucleic acid inserted into a desired site in the genome thereof thereby deleting the coding region of a desired endogenous target gene, *i.e.*, a knock-out transgenic mammal. Further, the invention includes a transgenic non-human mammal wherein an exogenous nucleic acid encoding REMODELIN is inserted into a site the genome, *i.e.*, a "knock-in" transgenic mammal. The knock-in transgene inserted may comprise various nucleic acids encoding, for example, a tag polypeptide, a promoter/regulatory region operably linked to the nucleic acid encoding REMODELIN not normally present in the cell or not typically operably linked to REMODELIN.

The generation of the non-human transgenic mammal of the invention is preferably accomplished using the method which is now described. However, the invention should in no way be construed as being limited solely to the use of this method, in that, other methods can be used to generate the desired knock-out mammal.

In the preferred method of generating a non-human transgenic mammal, ES cells are generated comprising the transgene of the invention and the cells are then used to generate the knock-out animal essentially as described in Nagy and Rossant (1993, In: Gene Targeting, A Practical Approach, pp.146-179, Joyner ed., IRL Press). ES cells behave as normal embryonic cells if they are returned to the embryonic environment by injection into a host blastocyst or aggregate with blastomere stage embryos. When so returned, the cells have the full potential to develop along all lineages of the embryo. Thus, it is possible, to obtain ES cells, introduce a desired

DNA therein, and then return the cell to the embryonic environment for development into mature mammalian cells, wherein the desired DNA may be expressed.

Precise protocols for the generation of transgenic mice are disclosed in Nagy and Rossant (1993, In: Gene Targeting, A Practical Approach, Joyner ed. IRL Press, pp. 146-179). and are therefore not repeated herein. Transfection or transduction of ES cells in order to introduce the desired DNA therein is accomplished using standard protocols, such as those described, for example, in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Preferably, the desired DNA contained within the transgene of the invention is electroporated into ES cells, and the cells are propagated as described in Soriano et al. (1991, Cell 64:693-702).

Introduction of an isolated nucleic acid into the fertilized egg of the mammal is accomplished by any number of standard techniques in transgenic technology (Hogan et al., 1986, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, NY). Most commonly, the nucleic acid is introduced into the embryo by way of microinjection.

Once the nucleic acid is introduced into the egg, the egg is incubated for a short period of time and is then transferred into a pseudopregnant mammal of the same species from which the egg was obtained as described, for example, in Hogan et al. (1986, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, NY). Typically, many eggs are injected per experiment, and approximately two-thirds of the eggs survive the procedure. About twenty viable eggs are then transferred into pseudopregnant animals, and usually four to ten of the viable eggs so transferred will develop into live pups.

Any mammalian REMODELIN gene may be used in the methods described herein to produce a transgenic mammal or a transgenic cell harboring a transgene comprising a deletion of all or part of that mammalian REMODELIN gene. Preferably, a rodent REMODELIN gene such as, *e.g.*, rat REMODELIN (SEQ ID NO:1), encoding rat REMODELIN$_S$ (SEQ ID NO:2) and rat REMODELIN$_L$ (SEQ ID NO:5), is used, and human REMODELIN (SEQ ID NO:3) gene, is also used.

The transgenic mammal of the invention can be any species of mammal. Thus, the invention should be construed to include generation of transgenic mammals encoding the chimeric nucleic acid, which mammals include mice, hamsters, rats, rabbits, pigs, sheep and cattle. The methods described herein for generation of transgenic mice can be analogously applied using any mammalian species. Preferably, the transgenic mammal of the invention is a rodent and even more preferably, the transgenic mammal of the invention is a mouse. By way of example, Lukkarinen et al. (1997, Stroke 28:639-645), teaches that gene constructs which enable the generation of transgenic mice also enable the generation of other transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus of an animal of one species can be replicated in an animal of another species having a genetic locus highly homologous to the first species.

To identify the transgenic mammals of the invention, pups are examined for the presence of the isolated nucleic acid using standard technology such as Southern blot hybridization, PCR, and/or RT-PCR. Expression of the nucleic acid in the cells and in the tissues of the mammal is also assessed using ordinary technology described herein. Further, the presence or absence of REMODELIN in the circulating blood of the transgenic animal can be determined, if the protein is secreted, by using, for example, Western blot analysis, or using standard methods for protein detection that are well-known in the art.

Cells obtained from the transgenic mammal of the invention, which are also considered "transgenic cells" as the term is used herein, encompass such as cells as those obtained from the REMODELIN (+/-) and (-/-) transgenic non-human mammal described elsewhere herein, are useful systems for modeling diseases and symptoms of mammals which are believed to be associated with altered levels of REMODELIN expression such as negative remodeling, arterial restenosis, adventitial fibrosis, wound healing, bone formation, bone density, dorsal closure, *spina bifida*-like conditions, and any other disease, disorder or condition associated with an altered level of REMODELIN expression. Moreover, as a marker of a pathway(s) associated with cell proliferation and cell migration, REMODELIN expression levels are also useful indicators in assessment of various diseases, disorders or conditions associated with

excessive or impaired wound healing (*e.g.*, skin wound healing) and conditions associated with excessive or impaired bone formation, and the like.

Particularly suitable are cells derived from a tissue of the non-human knock-out or knock-in transgenic mammal described herein, wherein the transgene comprising the REMODELIN gene is expressed or inhibits expression of REMODELIN in various tissues. By way of example, cell types from which such cells are derived include fibroblasts and like cells of (1) the REMODELIN (+/+), (+/-) and (-/-) non-human transgenic liveborn mammal, (2) the REMODELIN (+/+), (-/-) or (+/-) fetal animal, and (3) placental cell lines obtained from the REMODELIN (+/+), (-/-) and (+/-) fetus and liveborn mammal.

One skilled in the art would appreciate, based upon this disclosure, that cells comprising decreased levels of REMODELIN protein, decreased level of REMODELIN activity, or both, include, but are not limited to, cells expressing inhibitors of REMODELIN expression (*e.g.*, antisense or ribozyme molecules).

Methods and compositions useful for maintaining mammalian cells in culture are well known in the art, wherein the mammalian cells are obtained from a mammal including, but not limited to, cells obtained from a mouse such as the transgenic mouse described herein.

The recombinant cell of the invention can be used to study the effect of qualitative and quantitative alterations in REMODELIN levels on cell signal transduction systems. This is because the fact that the data disclosed herein indicate that REMODELIN is involved in TGF-β signaling pathways. Further, the recombinant cell can be used to produce REMODELIN for use for therapeutic and/or diagnostic purposes. That is, a recombinant cell expressing REMODELIN can be used to produce large amounts of purified and isolated REMODELIN that can be administered to treat or alleviate a disease, disorder or condition associated with or caused by a decreased level of REMODELIN.

Alternatively, recombinant cells expressing REMODELIN can be administered in *ex vivo* and *in vivo* therapies where administering the recombinant cells thereby administers the protein to a cell, a tissue, and/or an animal. Additionally, the

recombinant cells are useful for the discovery of REMODELIN ligand(s) and REMODELIN signaling pathway(s).

The recombinant cell of the invention may be used to study the effects of elevated or decreased REMODELIN levels on cell homeostasis and cell proliferation and/or migration since REMODELIN has been hypothesized to play a role in cell migration, adventitial fibrosis, arterial restenosis, negative remodeling, and the like

The recombinant cell of the invention, wherein the cell has been engineered such that it does not express REMODELIN, or expresses reduced or altered REMODELIN lacking biological activity, can also be used in *ex vivo* and *in vivo* cell therapies where either an animal's own cells (*e.g.*, fibroblasts, and the like), or those of a syngeneic matched donor, are recombinantly engineered as described elsewhere herein (*e.g.*, by insertion of an antisense nucleic acid or a knock-out vector such that REMODELIN expression and/or protein levels are thereby reduced in the recombinant cell), and the recombinant cell is administered to the recipient animal. In this way, recombinant cells that express REMODELIN at a reduced level can be administered to an animal whose own cells express increased levels of REMODELIN thereby treating or alleviating a disease, disorder or condition associated with or mediated by increased REMODELIN expression as disclosed elsewhere herein.

The transgenic mammal of the invention, rendered susceptible to adventitial fibrosis, arterial restenosis, and the like, such as, for example, a REMODELIN knock-out mouse, can be used to study the pathogenesis of these diseases and the potential role of REMODELIN therein.

Further, the transgenic mammal and/or cell of the invention may be used to further study the subcellular localization of REMODELIN.

Also, the transgenic mammal (both +/- and -/- live born and fetuses) and/or cell of the invention may be used to study to role(s) of REMODELIN in cell migration and proliferation, and TGF-β signaling to elucidate the target(s) of REMODELIN action as well as any receptor(s) and/or ligands that bind with REMODELIN to mediate its effect(s) in the cell.

VI. Antibodies

The invention also includes an antibody that specifically binds REMODELIN, or a fragment thereof.

One skilled in the art would understand, based upon the disclosure provided herein, that an antibody that specifically binds REMODELIN, binds with a protein of the invention, such as, but not limited to rat REMODELIN$_S$, human REMODELIN, and rat REMODELIN$_L$, or an immunogenic portion thereof. In one embodiment, the antibody is directed to rat REMODELIN comprising the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:5, and an antibody directed to human REMODELIN, comprising the amino acid sequence SEQ ID NO:4.

Polyclonal antibodies are generated by immunizing rabbits according to standard immunological techniques well-known in the art (*see, e.g.*, Harlow et al., 1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY). Such techniques include immunizing an animal with a chimeric protein comprising a portion of another protein such as a maltose binding protein or glutathione (GSH) tag polypeptide portion, and/or a moiety such that the REMODELIN portion is rendered immunogenic (*e.g.*, REMODELIN conjugated with keyhole limpet hemocyanin, KLH) and a portion comprising the respective rodent and/or human REMODELIN amino acid residues. The chimeric proteins are produced by cloning the appropriate nucleic acids encoding REMODELIN (*e.g.*, SEQ ID NO:1 and SEQ ID NO:3) into a plasmid vector suitable for this purpose, such as but not limited to, pMAL-2 or pCMX.

However, the invention should not be construed as being limited solely to these antibodies or to these portions of the protein antigens. Rather, the invention should be construed to include other antibodies, as that term is defined elsewhere herein, to rat and human REMODELIN, or portions thereof. Further, the present invention should be construed to encompass antibodies, *inter alia*, bind with REMODELIN and they are able to bind REMODELIN present on Western blots, in immunohistochemical staining of tissues thereby localizing REMODELIN in the tissues, and in immunofluorescence microscopy of a cell transiently transfected with a nucleic acid encoding at least a portion of REMODELIN.

One skilled in the art would appreciate, based upon the disclosure provided herein, that the antibody can specifically bind with any portion of the protein and the full-length protein can be used to generate antibodies specific therefor. However, the present invention is not limited to using the full-length protein as an immunogen. Rather, the present invention includes using an immunogenic portion of the protein to produce an antibody that specifically binds with mammalian REMODELIN. That is, the invention includes immunizing an animal using an immunogenic portion, or antigenic determinant, of the REMODELIN protein. Such immunogenic portions can include, but are not limited to, the carboxy-terminal 15 amino acids (GWNSVSRIIIEELPK) (SEQ ID NO:7). The antibodies can be produced by immunizing an animal such as, but not limited to, a rabbit or a mouse, with a protein of the invention, or a portion thereof, or by immunizing an animal using a protein comprising at least a portion of REMODELIN, or a fusion protein including a tag polypeptide portion comprising, for example, a maltose binding protein tag polypeptide portion covalently linked with a portion comprising the appropriate REMODELIN amino acid residues. One skilled in the art would appreciate, based upon the disclosure provided herein, that smaller fragments of these proteins can also be used to produce antibodies that specifically bind REMODELIN.

One skilled in the art would appreciate, based upon the disclosure provided herein, that various portions of an isolated REMODELIN polypeptide can be used to generate antibodies to either highly conserved regions of REMODELIN or to non-conserved regions of the polypeptide. As disclosed elsewhere herein, REMODELIN comprises various conserved domains including, but not limited to, a putative signal peptide from about amino acid residue 1 to about amino acid residue 32 transmembrane domain/signal peptide (amino acid residues from about 1 to 32); a CK2 phosphorylation domain (amino acid residues from about 31 to 34); an N-myristoylation domain (amino acid residues from about 69 to 74); a CK2 phosphorylation domain (amino acid residues from about 99 to 102); an N-myristoylation domain (amino acid residues from about 119 to 124); a PKC phosphorylation domain (amino acid residues from about 146 to 148); an N-myristoylation domain (amino acid residues from about 165 to 170); an N-

glycosylation domain (amino acid residues from about 188 to 191); a CK2 phosphorylation domain (amino acid residues from about 197 to 200); an N-myristoylation domain (amino acid residues from about 201 to 206); an N-myristoylation domain (amino acid residues from about 205 to 210); and a CK2 phosphorylation domain (amino acid residues from about 219 to 222). These domains are also present in rat and human REMODELINs (*see, e.g.*, SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5).

Once armed with the sequence of REMODELIN and the detailed analysis localizing the various conserved and non-conserved domains of the protein, the skilled artisan would understand, based upon the disclosure provided herein, how to obtain antibodies specific for the various portions of a mammalian REMODELIN polypeptide using methods well-known in the art or to be developed, as well as methods disclosed herein.

Further, the skilled artisan, based upon the disclosure provided herein, would appreciate that the non-conserved regions of a protein of interest can be more immunogenic than the highly conserved regions which are conserved among various organisms. Further, immunization using a non-conserved immunogenic portion can produce antibodies specific for the non-conserved region thereby producing antibodies that do not cross-react with other proteins which can share one or more conserved portions. Thus, one skilled in the art would appreciate, based upon the disclosure provided herein, that the non-conserved regions of each REMODELIN molecule can be used to produce antibodies that are specific only for that REMODELIN and do not cross-react non-specifically with other REMODELINs or with other proteins.

Alternatively, the skilled artisan would also understand, based upon the disclosure provided herein, that antibodies developed using a region that is conserved among one or more REMODELIN molecule can be used to produce antibodies that react specifically with one or more REMODELIN molecule. Methods for producing antibodies that specifically bind with a conserved protein domain which may otherwise be less immunogenic than other portions of the protein are well-known in the art and include, but are not limited to, conjugating the protein fragment of interest to a molecule (*e.g.*, keyhole limpet hemocyanin, and the like), thereby rendering the protein

domain immunogenic, or by the use of adjuvants (*e.g.*, Freund's complete and/or incomplete adjuvant, and the like), or both. Thus, the invention encompasses antibodies that recognize at least one REMODELIN and antibodies that specifically bind with more than one REMODELIN, including antibodies that specifically bind with all REMODELIN.

One skilled in the art would appreciate, based upon the disclosure provided herein, which portions of REMODELIN are less homologous with other proteins sharing conserved domains. However, the present invention is not limited to any particular domain; instead, the skilled artisan would understand that other non-conserved regions of the REMODELIN proteins of the invention can be used to produce the antibodies of the invention as disclosed herein.

Therefore, the skilled artisan would appreciate, based upon the disclosure provided herein, that the present invention encompasses antibodies that neutralize and/or inhibit REMODELIN activity (*e.g.*, by inhibiting necessary REMODELIN receptor/ligand interactions) which antibodies can recognize one or more REMODELINs, including, but not limited to, rat $REMODELIN_S$, rat $REMODELIN_L$, and human REMODELIN, as well as REMODELINs from various species (*e.g.*, mouse REMODELIN).

The invention should not be construed as being limited solely to the antibodies disclosed herein or to any particular immunogenic portion of the proteins of the invention. Rather, the invention should be construed to include other antibodies, as that term is defined elsewhere herein, to REMODELIN, or portions thereof, or to proteins sharing at least about 6% homology with a polypeptide having the amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5. Preferably, the polypeptide is about 10% homologous, more preferably, at least about 15% homologous, more preferably, at least about 20% homologous, even more preferably, at least about 25% homologous, more preferably, at least about 30% homologous, preferably, at least about 35% homologous, even more preferably, at least about 40% homologous, even more preferably, at least about 45% homologous, yet more preferably, at least about 50% homologous, more preferably, at least about 55% homologous, more preferably, at least about 60% homologous, even more preferably,

at least about 65% homologous, yet more preferably, at least about 70% homologous, more preferably, at least about 75% homologous, even more preferably, at least about 80% homologous, yet more preferably, at least about 85% homologous, more preferably, at least about 90% homologous, even more preferably, at least about 95% homologous, and most preferably, at least about 99% homologous to at least one of rat REMODELIN$_S$ (SEQ ID NO:2), human REMODELIN (SEQ ID NO:4), and rat REMODELIN$_L$ (SEQ ID NO:5). More preferably, the polypeptide that specifically binds with an antibody specific for mammalian REMODELIN is at least one of rat REMODELIN$_S$ (SEQ ID NO:2), human REMODELIN (SEQ ID NO:4), and rat REMODELIN$_L$ (SEQ ID NO:5). Most preferably, the polypeptide that specifically binds with an antibody that specifically binds with a mammalian REMODELIN is at least one of SEQ ID NO: 2, SEQ ID NO:4, and SEQ ID NO:5.

Further, the skilled artisan would appreciate, based upon the disclosure provided herein, that amino acid sequences that may elicit antibodies that non-specifically cross-react with a non-REMODELIN protein can also be excluded from use as immunogens. For example, such amino acid sequences include, but are not limited to, an amino acid sequence comprising collagen alpha-2 (IV) chain precursor (GenBank Acc. No. P27393), which shares about 62% identity with REMODELIN over a 35 amino acid stretch. Thus, such a portion sharing at least about 62% identity over 35 amino acids of REMODELIN would not be used to produce the antibodies of the invention.

The invention encompasses polyclonal, monoclonal, synthetic antibodies, and the like. One skilled in the art would understand, based upon the disclosure provided herein, that the crucial feature of the antibody of the invention is that the antibody bind specifically with REMODELIN. That is, the antibody of the invention recognizes REMODELIN, or a fragment thereof (*e.g.*, an immunogenic portion or antigenic determinant thereof), as demonstrated by antibody binding REMODELIN on Western blots, in immunostaining of cells, and/o immunoprecipitation of REMODELIN, using standard methods well-known in the art.

One skilled in the art would appreciate, based upon the disclosure provided herein, that the antibodies can be used to localize the relevant protein in a cell

and to study the role(s) of the antigen recognized thereby in cell processes. Moreover, the antibodies can be used to detect and or measure the amount of protein present in a biological sample using well-known methods such as, but not limited to, Western blotting and enzyme-linked immunosorbent assay (ELISA). Moreover, the antibodies can be used to immunoprecipitate and/or immuno-affinity purify their cognate antigen using methods well-known in the art.

In addition, the antibody can be used to decrease the level of REMODELIN in a cell thereby inhibiting the effect(s) of REMODELIN in a cell. Thus, by administering the antibody to a cell or to the tissues of an animal or to the animal itself, the required REMODELIN receptor/ligand interactions are therefore inhibited such that the effect of REMODELIN-mediated signaling are also inhibited. One skilled in the art would understand, based upon the disclosure provided herein, that detectable effects upon inhibiting REMODELIN ligand/receptor interaction using an anti-REMODELIN antibody can include, but are not limited to, decreased proliferation of cells, decreased cell migration, decreased negative modeling, decreased adventitial fibrosis, decreased arterial restenosis, decreased fibrosis in any organ or tissue, decreased ossification or bone formation, and the like.

The generation of polyclonal antibodies is accomplished by inoculating the desired animal with the antigen and isolating antibodies which specifically bind the antigen therefrom using standard antibody production methods such as those described in, for example, Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY).

Monoclonal antibodies directed against full length or peptide fragments of a protein or peptide may be prepared using any well known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY) and in Tuszynski et al. (1988, Blood, 72:109-115). Quantities of the desired peptide may also be synthesized using chemical synthesis technology. Alternatively, DNA encoding the desired peptide may be cloned and expressed from an appropriate promoter sequence in cells suitable for the generation of large quantities of peptide. Monoclonal antibodies

directed against the peptide are generated from mice immunized with the peptide using standard procedures as referenced herein.

Nucleic acid encoding the monoclonal antibody obtained using the procedures described herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. Immunol. 12:125-168), and the references cited therein.

Further, the antibody of the invention may be "humanized" using the technology described in, for example, Wright et al. (*supra*), and in the references cited therein, and in Gu et al. (1997, Thrombosis and Hematocyst 77:755-759), and other methods of humanizing antibodies well-known in the art or to be developed.

To generate a phage antibody library, a cDNA library is first obtained from mRNA which is isolated from cells, *e.g.*, the hybridoma, which express the desired protein to be expressed on the phage surface, *e.g.*, the desired antibody. cDNA copies of the mRNA are produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook et al., *supra*.

Bacteriophage which encode the desired antibody, may be engineered such that the protein is displayed on the surface thereof in such a manner that it is available for binding to its corresponding binding protein, *e.g.*, the antigen against which the antibody is directed. Thus, when bacteriophage which express a specific antibody are incubated in the presence of a cell which expresses the corresponding antigen, the bacteriophage will bind to the cell. Bacteriophage which do not express the antibody will not bind to the cell. Such panning techniques are well known in the art and are described for example, in Wright et al. (*supra*).

Processes such as those described above, have been developed for the production of human antibodies using M13 bacteriophage display (Burton et al., 1994, Adv. Immunol. 57:191-280). Essentially, a cDNA library is generated from mRNA obtained from a population of antibody-producing cells. The mRNA encodes

rearranged immunoglobulin genes and thus, the cDNA encodes the same. Amplified cDNA is cloned into M13 expression vectors creating a library of phage which express human Fab fragments on their surface. Phage which display the antibody of interest are selected by antigen binding and are propagated in bacteria to produce soluble human Fab immunoglobulin. Thus, in contrast to conventional monoclonal antibody synthesis, this procedure immortalizes DNA encoding human immunoglobulin rather than cells which express human immunoglobulin.

The procedures just presented describe the generation of phage which encode the Fab portion of an antibody molecule. However, the invention should not be construed to be limited solely to the generation of phage encoding Fab antibodies. Rather, phage which encode single chain antibodies (scFv/phage antibody libraries) are also included in the invention. Fab molecules comprise the entire Ig light chain, that is, they comprise both the variable and constant region of the light chain, but include only the variable region and first constant region domain (CH1) of the heavy chain. Single chain antibody molecules comprise a single chain of protein comprising the Ig Fv fragment. An Ig Fv fragment includes only the variable regions of the heavy and light chains of the antibody, having no constant region contained therein. Phage libraries comprising scFv DNA may be generated following the procedures described in Marks et al. (1991, J. Mol. Biol. 222:581-597). Panning of phage so generated for the isolation of a desired antibody is conducted in a manner similar to that described for phage libraries comprising Fab DNA.

The invention should also be construed to include synthetic phage display libraries in which the heavy and light chain variable regions may be synthesized such that they include nearly all possible specificities (Barbas, 1995, Nature Medicine 1:837-839; de Kruif et al. 1995, J. Mol. Biol. 248:97-105).

VII. Compositions

The invention includes a composition comprising an isolated nucleic complementary to a nucleic acid, or a portion thereof, encoding a mammalian REMODELIN, which is in an antisense orientation with respect to transcription. Preferably, the composition comprises a pharmaceutically acceptable carrier.

The invention includes a composition comprising an isolated mammalian REMODELIN polypeptide as described herein. Preferably, the composition comprises a pharmaceutically-acceptable carrier.

The invention also includes a composition comprising an antibody that specifically binds REMODELIN. Preferably, the composition comprises a pharmaceutically-acceptable carrier.

The invention further includes a composition comprising an isolated nucleic acid encoding a mammalian REMODELIN. Preferably, the composition comprises a pharmaceutically acceptable carrier.

The compositions can be used to administer REMODELIN to a cell, a tissue, or an animal or to inhibit expression of REMODELIN in a cell, a tissue, or an animal. The compositions are useful to treat a disease, disorder or condition mediated by altered expression of REMODELIN such that decreasing or increasing REMODELIN expression or the level of the protein in a cell, tissue, or animal, is beneficial to the animal. That is, where a disease, disorder or condition in an animal is mediated by or associate with altered level of REMODELIN expression or protein level, the composition can be used to modulate such expression or protein level of REMODELIN.

For administration to the mammal, a polypeptide, or a nucleic acid encoding it, and/or an antisense nucleic acid complementary to all or a portion thereof, can be suspended in any pharmaceutically acceptable carrier, for example, HEPES buffered saline at a pH of about 7.8.

Other pharmaceutically acceptable carriers which are useful include, but are not limited to, glycerol, water, saline, ethanol and other pharmaceutically acceptable salt solutions such as phosphates and salts of organic acids. Examples of these and other pharmaceutically acceptable carriers are described in Remington's Pharmaceutical Sciences (1991, Mack Publication Co., New Jersey).

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents,

wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides.

Pharmaceutical compositions that are useful in the methods of the invention may be administered, prepared, packaged, and/or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

The compositions of the invention may be administered via numerous routes, including, but not limited to, oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, or ophthalmic administration routes. The route(s) of administration will be readily apparent to the skilled artisan and will depend upon any number of factors including the type and severity of the disease being treated, the type and age of the veterinary or human patient being treated, and the like.

Pharmaceutical compositions that are useful in the methods of the invention may be administered systemically in oral solid formulations, ophthalmic, suppository, aerosol, topical or other similar formulations. In addition to the compound such as heparan sulfate, or a biological equivalent thereof, such pharmaceutical compositions may contain pharmaceutically-acceptable carriers and other ingredients known to enhance and facilitate drug administration. Other possible formulations, such as nanoparticles, liposomes, resealed erythrocytes, and immunologically based systems may also be used to administer REMODELIN and/or a nucleic acid encoding the same according to the methods of the invention.

Compounds which are identified using any of the methods described herein may be formulated and administered to a mammal for treatment of arterial restenosis, adventitial fibrosis, fibrosis in any organ or tissue, negative remodeling, excessive bone formation, excessive ossification, and the like, are now described.

The invention encompasses the preparation and use of pharmaceutical compositions comprising a compound useful for treatment of arterial restenosis, adventitial fibrosis, negative remodeling, and the like, as an active ingredient. Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. The active ingredient may be present in the pharmaceutical composition in the form of a physiologically acceptable ester or salt, such as in combination with a physiologically acceptable cation or anion, as is well known in the art.

As used herein, the term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient may be combined and which, following the combination, can be used to administer the active ingredient to a subject.

As used herein, the term "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient which is compatible with any other ingredients of the pharmaceutical composition, which is not deleterious to the subject to which the composition is to be administered.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to

which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, intrathecal or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents. Particularly contemplated additional agents include anti-emetics and scavengers such as cyanide and cyanate scavengers.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete

solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient. Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

As used herein, an "oily" liquid is one which comprises a carbon-containing liquid molecule and which exhibits a less polar character than water.

A tablet comprising the active ingredient may, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include, but are not limited to, potato starch and sodium starch glycollate. Known surface active agents include, but are not limited to, sodium lauryl sulphate. Known diluents include, but are not limited to, calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include, but are not limited to, corn starch and alginic acid. Known binding agents include, but are not limited to, gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include, but are not limited to, magnesium stearate, stearic acid, silica, and talc.

Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient. By way of example, a material such as glyceryl monostearate or glyceryl distearate may be used to

coat tablets. Further by way of example, tablets may be coated using methods described in U.S. Patents numbers 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Hard capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient, and may further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

Soft gelatin capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient, which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the active ingredient in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents

include, but are not limited to, naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (*e.g.*, polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para- hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The

oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient with a non-irritating pharmaceutically acceptable excipient which is solid at ordinary room temperature (*i.e.*, about 20°C) and which is liquid at the rectal temperature of the subject (*i.e.*, about 37°C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition may be in the form of, for example, a suppository, an impregnated or

coated vaginally-insertable material such as a tampon, a douche preparation, or gel or cream or a solution for vaginal irrigation.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (*i.e.*, such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject. Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a

preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (*i.e.*, powder or granular) form for reconstitution with a suitable vehicle (*e.g.*, sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as

methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close to the nares.

Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, 0.1 to 20% (w/w) active ingredient, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient. Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient in an aqueous or oily liquid carrier. Such drops may further comprise buffering agents, salts, or one or more

other of the additional ingredients described herein. Other ophthalmalmically-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form or in a liposomal preparation.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed. (1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA), which is incorporated herein by reference.

Typically, dosages of the compound of the invention which may be administered to an animal, preferably a human, will vary depending upon any number of factors, including but not limited to, the type of animal and type of disease state being treated, the age of the animal and the route of administration.

The compound can be administered to an animal as frequently as several times daily, or it may be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even lees frequently, such as once every several months or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity of the disease being treated, the type and age of the animal, etc.

VIII. Methods

A. Methods of identifying useful compounds

The present invention further includes a method of identifying a compound that affects expression of REMODELIN in a cell. The method comprises contacting a cell with a test compound and comparing the level of expression of REMODELIN in the cell so contacted with the level of expression of REMODELIN in an otherwise identical cell not contacted with the compound. If the level of expression of REMODELIN is higher or lower in the cell contacted with the test compound compared to the level of expression of REMODELIN in the otherwise identical cell not contacted with the test compound, this is an indication that the test compound affects expression of REMODELIN in a cell.

Similarly, the present invention includes a method of identifying a compound that reduces expression of REMODELIN in a cell. The method comprises contacting a cell with a test compound and comparing the level of expression of REMODELIN in the cell contacted with the compound with the level of expression of REMODELIN in an otherwise identical cell, which is not contacted with the compound. If the level of expression of REMODELIN is lower in the cell contacted with the compound compared to the level in the cell that was not contacted with the compound, then that is an indication that the test compound affects reduces expression of REMODELIN in a cell.

One skilled in the art would appreciate, based on the disclosure provided herein, that the level of expression of REMODELIN in the cell can be measured by determining the level of expression of mRNA encoding REMODELIN. Alternatively, the level of expression of mRNA encoding REMODELIN can be determined by using immunological methods to assess REMODELIN production from such mRNA as exemplified herein using Western blot analysis using an anti-REMODELIN antibody of the invention. Further, nucleic acid-based detection methods, such as Northern blot and PCR assays and the like, can be used as well. In addition, the level of REMODELIN activity in a cell can also be assessed by determining the level of various parameters which can be affected by REMODELIN activity such as, for example, the level of cell proliferation and/or migration, the level of expression in adventitia, the level of adventitial fibrosis, the level of fibrosis in other organs (*e.g.*, lung, liver, among others), the level of arterial restenosis, the level of

ossification, the level of bone formation and fracture healing, the level of osteoblast proliferation, and the like. Thus, one skilled in the art would appreciate, based upon the extensive disclosure and reduction to practice provided herein, that there are a plethora of methods which can be used to asses the level of expression of REMODELIN in a cell including those methods disclosed herein, methods well-known in the art, and other methods to be developed in the future.

Further, one skilled in the art would appreciate based on the disclosure provided herein that, as disclosed in the examples below, a cell which lacks endogenous REMODELIN expression can be transfected with a vector comprising an isolated nucleic acid encoding REMODELIN whereby expression of REMODELIN is effected in the cell. The transfected cell is then contacted with the test compound thereby allowing the determination of whether the compound affects the expression of REMODELIN. Therefore, one skilled in the art armed with the present invention would be able to, by selectively transfecting a cell lacking detectable levels of REMODELIN using REMODELIN-expressing vectors, identify a compound which selectively affects REMODELIN expression.

The skilled artisan would further appreciate, based upon the disclosure provided herein, that where an isolated nucleic acid encoding REMODELIN is administered to a cell lacking endogenous detectable levels of REMODELIN expression such that detectable REMODELIN is produced by the cell, the isolated nucleic acid can comprise an additional nucleic acid encoding, *e.g.*, a tag polypeptide, covalently linked thereto. This allows the detection of REMODELIN expression by detecting the expression of the tag polypeptide. Thus, the present invention encompasses methods of detecting REMODELIN expression by detecting expression of another molecule which is co-expressed with REMODELIN.

The invention includes a method of identifying a protein that specifically binds with REMODELIN. That is, one skilled in the art would appreciate, based upon the disclosure provided herein, that REMODELIN, which comprises several myristoylation domains. Further, REMODELIN comprises a putative signal peptide indicating the molecule can be secreted from a cell. These data indicate that REMODELIN likely effects its biological function(s) by specifically binding with at

least one protein, preferably a REMODELIN receptor, another REMODELIN molecule, and/or a REMODELIN ligand. Thus, the invention encompasses methods, which are well-known in the art or to be developed, for identifying a protein that specifically binds with and/or associates with REMODELIN. Such methods include, but are not limited to, protein binding assays wherein the target protein, *i.e.*, REMODELIN, is immobilized on an appropriate support and incubated under conditions that allow REMODELIN binding with a REMODELIN-associated protein. REMODELIN can be immobilized on a support using standard methods such as, but not limited to, production of REMODELIN comprising a glutathione-S-transferase (GST) tag, a maltose binding protein (MBP) tag, or a His_6-tag, where the fusion protein is then bound to glutathione-Sepharose beads, a maltose-column, or a nickel chelation resin (*e.g.*, His-Bind resin, Novagen, Madison, WI), respectively. The solid support is washed to remove proteins which may be non-specifically bound thereto and any REMODELIN-associated protein can then be dissociated from the matrix thereby identifying a REMODELIN-associated protein.

In addition, a protein that specifically binds with REMODELIN, *e.g.*, a receptor, a ligand, and/or other REMODELIN-associated protein, can be identified using, for example, a yeast two hybrid assay. Yeast two hybrid assay methods are well-known in the art and can be performed using commercially available kits (*e.g.*, MATCHMAKER™ Systems, Clontech Laboratories, Inc., Palo Alto, CA, and other such kits) according to standard methods. Therefore, once armed with the teachings provided herein, *e.g.*, the full amino and nucleic acid sequences of the "bait" protein, REMODELIN, one skilled in the art can easily identify a protein that specifically binds with REMODELIN such as, but not limited to, a REMODELIN receptor protein, a REMODELIN ligand, and the like.

One skilled in the art would understand, based upon the disclosure provided herein, that the invention encompasses any molecule identified using the methods discussed elsewhere herein. That is, molecules that associate with REMODELIN, such as but not limited to, a REMODELIN receptor protein, a REMODELIN ligand protein, or both, can be used to develop therapeutics and diagnostics for diseases, disorders or conditions mediated by REMODELIN interaction

with a REMODELIN-associated protein such as negative remodeling, arterial restenosis, adventitial fibrosis, excessive wound healing responses, scarring, keloids, excessive bone formation, fracture healing, ectopic ossification, excessive fibrous tissue formation, failure of dorsal closure, *spina bifida*-like effects, and the like. That is, one skilled in the art would appreciate, as more fully set forth elsewhere herein in discussing antibodies that specifically bind with REMODELIN, that a REMODELIN-associated protein can be used to develop therapeutics that inhibit REMODELIN activity in a cell by inhibiting necessary REMODELIN receptor/ligand interactions and other REMODELIN binding interactions, which are required for REMODELIN activity.

REMODELIN-associated proteins identified by the above-disclosed methods can be used directly to inhibit REMODELIN interactions by contacting a cell with the REMODELIN-associated protein, or a portion thereof, or they can be used to develop antibodies and/or peptidomimetics that can inhibit the REMODELIN-associated interaction with REMODELIN thereby inhibiting REMODELIN function and activity. Thus, REMODELIN-associated proteins, including a REMODELIN receptor/ligand protein, are useful and are encompassed by the invention.

B. Methods of treating or alleviating a disease, disorder or condition associated with or mediated by REMODELIN expression

The invention includes a method of alleviating a disease, disorder or condition mediated by altered expression of REMODELIN. The method comprises administering an antisense nucleic acid complementary to a nucleic acid encoding REMODELIN to a patient afflicted with a disease, disorder or condition mediated by increased REMODELIN expression compared to the level of REMODELIN expression in otherwise identical but normal tissue, *i.e.*, tissue which does not exhibit any detectable clinical parameters associated with the disease, disorder or condition being treated or alleviated. This, in turn, mediates a decrease in REMODELIN expression thereby alleviating a disease, disorder or condition mediated by abnormal expression of REMODELIN. Such diseases, disorder or conditions include, but are not limited to, negative remodeling, arterial restenosis, adventitial fibrosis, excessive wound healing

responses, scarring, keloids, abnormal bone formation and/or bone density, fracture healing, ectopic ossification, excessive fibrous tissue formation, lack of dorsal closure, *spina bifida*-like conditions, osteogenesis imperfecta (OI), dystrophic epidermolysis bullosea (DEB), Bethlem myopathy, tissue calcification, including, but not limited to, calcification of implants, such as heart valves, and the like.

The data disclosed herein demonstrate that REMODELIN expression is induced in fibroblasts following an injury. As such, REMODELIN is part of any wound healing process which is characterized by granulation tissue formation, proliferation, and migration of fibroblasts with subsequent apoptosis of these cells extracellular matrix production. Although the wound healing response/process is a normal physiologic response to injury, there are many conditions where an excessive wound healing response leads to symptoms or disease. Thus, the skilled artisan would appreciate, based upon the disclosure provided herein, that because the wound healing response is similar for all organs and tissues, the effects of expression of REMODELIN are similar independent of where the injury occurred. Excessive wound healing would result in various conditions depending on the situs of injury such that, for example, in an artery, excessive wound healing would result in negative remodeling with loss of vessel diameter due to adventitial fibrosis. The formation of scars and keloids is an excessive fibrotic reaction associated with excessive wound healing. Chronic inflammatory conditions often lead to organ fibrosis, *e.g.*, liver fibrosis and lung fibrosis.

The data disclosed herein demonstrate that when REMODELIN is inhibited using, for example, but not limited to, an antisense nucleic acid, the cell exhibited an altered morphology indicating decreased cell adhesion to the substratum and decreased cell-cell interaction. Further, the data disclosed herein demonstrate that REMODELIN antisense expression is associated with and/or mediates cell turnover. Therefore, the data further indicate that REMODELIN is involved in and/or is associated with processes involving cell turnover, cell adhesion and cell-cell interaction such as, but not limited to, negative remodeling, adventitial fibrosis, and the like.

An example of a disease, disorder or a condition associated with or mediated by REMODELIN expression is, organ and tissue fibrosis (*e.g.*, adventitial,

lung, liver, and retroperitoneal fibrosis, and the like), hypertrophic scars, keloids, excessive bone formation, ectopic ossification, and the like.

Although inhibition of REMODELIN is exemplified by administering an antisense to a cell thereby inhibiting expression of REMODELIN in the cell, one skilled in the art would appreciate that there are a wide plethora of methods for inhibiting protein expression and/or activity in a cell. Such methods include, but are not limited to, inhibiting expression of REMODELIN using ribozymes, and inhibiting activity of the protein in a cell by, for instance, administering an antibody to the cell by, *e.g.*, administering a nucleic acid encoding the antibody to the cell such that the antibody is expressed in the cell thus delivering the antibody to the cell cytosol. The use of these "intrabodies" to inhibit the intracellular activity of a protein are well-known in the art. *See, e.g.*, Verma et al. (1997, Nature 389:239-242; Benhar & Pastan, 1995, J. Biol. Chem. 270:23373-23380; Willuda et al., 1999, Cancer Res. 59:5758-5767; and Worn et al., 2000, J. Biol. Chem. 275:2795-2803). Therefore, the present invention encompasses any method of inhibiting the activity of a protein of interest in a cell using such methods as are known in the art or to be developed in the future.

The invention includes a method of treating bone disease in a mammal by inhibiting or decreasing expression of REMODELIN. This is inhibiting e the data disclosed herein demonstrate that inhibiting REMODELIN in osteoblasts mediates a powerful effect thereby suggesting a role for this gene in bone formation and indicating a role for REMODELIN in certain bone disease. That is, bone disease can be mediated by and/or associated with bone formation at undesirable sites, such as after trauma, leading to ossification of otherwise non-calcifying tissues, for example, skeletal muscle and vascular tissues. Indeed, calcification is an important problem involving prostheses and implants, such as, but not limited to, heart valves. Therefore, the data disclosed herein suggest that REMODELIN expression plays a role in bone formation, ossification, and calcification in response to trauma and/or injury such that methods affecting that expression can treat a disease, such as a bone disease, mediated by or associated with those REMODELIN-associated processes.

The data presented herein further indicates that over-expression of REMODELIN mediates and/or is associated with lack of dorsal closure, abnormal

bone density and bone growth, including dwarfism, and *spina bifida*-like phenotype. Thus, these data demonstrate that a method of inhibiting or decreasing REMODELIN expression can be useful for treating diseases associated with or mediated by over-expression of REMODELIN such as, but not limited to, treatment of lack of dorsal closure, abnormal bone density, dwarfism, *spina bifida*-like phenotype, and the like. Thus, the data disclosed herein suggest that REMODELIN plays an important role(s) including, but not limited to, a role in bone formation, bone density, dorsal closure, inhibition of calcification, and the like, and that any disease, disorder or condition associated with those processes can be treated or alleviated by any treatment decreasing or inhibiting the level of REMODELIN expression compared with the level of REMODELIN in the absence of such treatment.

One skilled in the art would appreciate, based upon the disclosure provided herein, that conditions associated with premature calcification or mineralization of bone and other tissues, including vascular calcification, and calcification of transplants, including, but not limited to, heart valves, can be treated or ameliorated by inhibiting REMODELIN expression. This is because the data disclosed herein demonstrate that REMODELIN expression is associated with, or mediates, bone mineralization and calcification such that inhibiting such expression can inhibit those processes. Inhibition of calcification provides an important therapeutic benefit for treatment of conditions associated with, or mediated by, premature ossification or unwanted calcification generally, such conditions being well-known in the art.

The invention includes a method of treating cartilage disease in a mammal. This is because, as demonstrated by the data disclosed elsewhere herein, lack of REMODELIN expression can lead to premature calcification of the cartilage matrix thereby inhibiting bone growth. Thus, the present invention encompasses a method of treating cartilage disease wherein the level of REMODELIN in a cell, tissue or organ in a patient suffering from cartilage disease, is increased relative to the level of REMODELIN in a cell from the patient prior to, or in the absence of, treatment or detectably increased with respect to the level of REMODELIN in an otherwise identical cell obtained from a mammal not suffering from any disease, disorder or

condition. Therefore, the present invention includes methods to treat cartilage disease by increasing REMODELIN expression.

One skilled in the art would understand, based upon the disclosure provided herein, that reducing expression of REMODELIN can mediate a beneficial effect in a patient afflicted with excessive wound healing resulting in negative remodeling, adventitial fibrosis, fibrosis in any organ or tissue (*e.g.*, liver fibrosis and lung fibrosis), scarring, keloids, fibrotic reaction associated with excessive wound healing, excessive bone formation, ectopic ossification, and the like. Thus, decreased REMODELIN expression can be useful for treating such diseases, disorders, or conditions, and the present invention encompasses such methods of treating these diseases by inhibiting the level of REMODELIN in a cell. This is because, as disclosed elsewhere herein, increased expression of REMODELIN is associated with abnormal cell proliferation associated with arterial restenosis, negative remodeling, adventitial fibrosis, fibrosis in any organ or tissue (*e.g.*, liver, lung, among others), excessive bone formation, ectopic ossification, altered cell adhesion and cell-cell interaction, cell turnover, and the like. Thus, one skilled in the art would appreciate, based upon the disclosure provided herein, that inhibition of REMODELIN expression can inhibit the deleterious effects of REMODELIN abnormal expression.

One skilled in the art would understand, based upon the disclosure provided herein, that since reduced REMODELIN expression can mediate a beneficial effect, methods of decreasing expression of REMODELIN, decreasing the stability of the protein in the cytoplasm, decreasing the level of REMODELIN polypeptide present in the cell, and/or decreasing the activity of REMODELIN in a cell (using, *e.g.*, antisense nucleic acids, ribozymes, antibodies, and the like), can be used to treat and/or alleviate a disease, disorder or condition associated with altered expression of REMODELIN where a lower level of REMODELIN relative to the level of REMODELIN in that cell prior to, or in the absence of, treatment provides a benefit. A benefit such as preventing decreased bone growth and/or density, failure of dorsal closure, and *spina bifida*-like phenotype such as are associated with over-expression of REMODELIN as disclosed elsewhere herein. Thus, whether an antisense nucleic acid or a blocking antibody is administered, the present invention includes a method where

the expression of REMODELIN be reduced in a cell compared with an otherwise cell, to treat/or alleviate preventing decreased bone growth and/or density, failure of dorsal closure, and *spina bifida*-like phenotype.

Techniques for inhibiting expression of a nucleic acid in a cell are well known in the art and encompass such methods as disclosed herein (*e.g.*, inhibition using an antibody, an antisense nucleic acid, and the like). Other techniques useful for inhibiting expression of a nucleic acid encoding REMODELIN include, but are not limited to, using nucleotide reagents that target specific sequences of the REMODELIN promoter, and the like.

Antisense nucleic acids that inhibit expression of REMODELIN, among other things, can be used for the manufacture of a medicament for treatment of a disease, disorder or condition mediated by increased expression of REMODELIN, when compared with expression of REMODELIN in a cell and/or a patient not afflicted with the disease, disorder or condition.

One skilled in the art would understand, based upon the disclosure provided herein, that it may be useful to increase the level or activity of REMODELIN in a cell. That is, the data disclosed herein demonstrating the association between REMODELIN expression and wound healing and bone growth, indicate that overexpression or an increase in REMODELIN activity can accelerate wound healing and bone growth. This can be useful to treat or alleviate a disease, disorder of condition associated with or mediated by decreased expression, level, or activity of REMODELIN (*e.g.*, various forms of organ and tissue damage including bone fractures), when compared to the expression, level or activity of REMODELIN in otherwise identical cell, tissue, or animal that does not suffer from the disease, disorder or condition, by administering REMODELIN. Such diseases, disorders or conditions include, but are not limited to, tissue damage or injury, including bone fracture, wound healing, and the like, and collagen-related diseases.

The data disclosed herein suggest that over- and under-expression of REMODELIN are associated with disease, disorder or condition such that a method of decreasing or increasing the level of REMODELIN can produce a benefit. Therefore, a method of affecting the level of REMODELIN to treat/alleviate a wide plethora of

diseases, disorders, or conditions is disclosed herein, where the level of REMODELIN is either decreased or increased compared with the level of REMODELIN in the cell prior to treatment, or compared with the level of REMODELIN in an otherwise identical cell that is obtained from a mammal known not to be afflicted with a disease, disorder or condition associated with, or mediated by, an altered level of REMODELIN.

The skilled artisan would understand, based upon the disclosure provided herein, that both increasing and decreasing REMODELIN expression and/or activity in a cell, tissue or organ can treat a disease, disorder or condition associated with REMODEL expression or activity. This is because the data disclosed herein demonstrate that in certain instances, decreasing REMODELIN expression provides a benefit while, in other instances, increasing REMODELIN expression is therapeutic, *e.g.*, by accelerating wound healing, bone formation, and the like. Therefore, the present invention encompasses methods of treating and/or alleviating conditions associated with either increased or decreased REMODELIN expression, and the skilled artisan, armed with the disclosure provided herein, would appreciate which conditions would benefit from decreasing or increasing REMODELIN expression and/or activity in a cell in a mammal.

Whether expression of REMODELIN, levels of the polypeptide, or its activity, is increased or decreased, one skilled in the art would appreciate, based on this disclosure, that methods of reducing or inducing REMODELIN of the invention encompass administering a recombinant cell that either expresses or lacks expression of REMODELIN. Thus, one skilled in the art would appreciate, based on the disclosure provided herein, that the present invention encompasses cell and gene therapy methods to effect either a detectable increase or decrease in the level of REMODELIN expression in a mammal.

In another embodiment of the invention, an individual suffering from a disease, disorder or a condition that is associated with or mediated by REMODELIN expression can be treated by supplementing, augmenting and/or replacing defective cells with cells that lack REMODELIN expression. The cells can be derived from cells obtained from a normal syngeneic matched donor or cells obtained from the individual

to be treated. The cells may be genetically modified to inhibit REMODELIN expression. Alternatively, the cells can be modified to increase REMODELIN expression using recombinant methods well-known in the art. Also, the invention encompasses using normal cells obtained from an otherwise identical donor that does not suffer from any disease or disorder associated with altered REMODELIN expression, which cells can be administered to a mammal in need thereof.

Additionally, the invention includes *ex vivo* techniques where a cell is obtained from the mammal, modified to express increased or decreased level of REMODELIN, and reintroduced into the mammal. Moreover, cells from the mammal which express a normal level of REMODELIN, compared with the level of REMODELIN expressed in an otherwise identical cell obtained from a like mammal not suffering from any condition associated with altered REMODELIN expression, can be grown and expanded and an effective number of the cells can be reintroduced into the mammal. Such methods include cell and gene therapy techniques relating to use of bone marrow stromal cells which methods are well-known in the art. Thus, one skilled in the art would appreciate that cell therapy and gene therapy relating to cells that have or lack detectable REMODELIN expression wherein the cells are administered *in vivo* are encompassed in the present invention.

The data presented herein demonstrate that REMODELIN expression can be modulated using TGF-β and TGF-β receptor. Specifically, TGF-β induces expression of REMODELIN, and TGF-β receptor reduces expression of REMODELIN perhaps by, and without wishing to be bound by any particular theory, competing for the free REMODELIN thereby inhibiting the ligand/receptor interaction between TGF-β and its receptor associated with an increase in the level of REMODELIN expression. Thus, one skilled in the art would understand, based upon the disclosure provided herein, that TGF-β or TGF-β receptor can be administered to a mammal to treat a disease, disorder or a condition associated with altered expression of REMODELIN in a mammal.

Accordingly, one skilled in the art, armed with the disclosure provided herein, would appreciate that the invention includes a method of increasing REMODELIN expression in a mammal by administering TGF-β. This is because, as

discussed previously elsewhere herein, and as would be appreciated by the routineer based upon the disclosure provided herein, interaction of TGF-β with its receptor, *i.e.*, TGF-β receptor type II, increases the level of REMODELIN expression in a cell contacted with TGF-β. Therefore, where increased level of REMODELIN expression provides a benefit, as more fully discussed elsewhere herein, administering TGF-β, which increases the level of REMODELIN expression compared with the level of REMODELIN expression prior to, or in the absence of, TGF-β, is desired and provides a therapeutic benefit.

Alternatively, one skilled in the art would understand, based upon the disclosure provided herein, that the invention includes a method of reducing REMODELIN expression in a mammal by administering TGF-β receptor type II. This is because, as noted previously elsewhere herein and without wishing to be bound by any particular theory, the data disclosed herein demonstrate that interaction between TGB-β and its receptor, TGF-β receptor II, mediates an increase in the level of REMODELIN expression in a cell such that inhibiting that interaction by administering free, soluble TGF-β receptor type II can reduce the level of interaction of TGF-β with a TGF-β receptor type II on the surface of a cell, thereby reducing and/or preventing the increase in the level of REMODELIN expression mediated by TGF-β that would otherwise occur in the absence of the excess receptor. Thus, administration of TGF-β receptor type II decreases the level of REMODELIN expression, and can be used to treat any disease, disorder or condition associated with, or mediated by, increased level of REMODELIN such as, but not limited to, ...

The skilled artisan would further appreciate, based upon the disclosure provided herein, that a TGF-β receptor type II antagonist is included in the invention. That is, any compound that specifically inhibits transmission of a signal via the TGF-β receptor type II can be used to inhibit expression of REMODELIN. Such TGF-β antagonists include, but are not limited to, any molecule that inhibits TGF-β interaction with the receptor such as an antibody that sterically inhibits ligand/receptor interaction between TGF-β and TGF-β receptor type II. Such molecules, by inhibiting necessary interaction between TGF-β and the TGF-β receptor type II, inhibit TGF-β-associated increase in REMODELIN expression thereby inhibiting the effect(s) of increased

REMODELIN expression in cell processes. TGF-β receptor type II antagonists are well-known in the art and the invention further encompasses such antagonists as are discovered in the future.

The skilled artisan would also understand, based upon the disclosure provided herein, that the amount of inhibitor administered to a mammal in order to inhibit REMODELIN expression can be easily assessed by determining the level of REMODELIN expression before and after administration of the inhibitor. Preferably, inhibition of REMODELIN expression mediated by TGF-β using soluble TGF-β receptor type II comprises administering about 2 mg/kg of soluble receptor; however, the present invention is not limited to this dose, and the skilled artisan would be able to readily determine, based upon the disclosure provided herein, the appropriate dose for each mammal depending on various parameters, including, but not limited to, the level of REMODELIN expression detected in the mammal prior to and during treatment.

The present invention encompasses increasing REMODELIN expression by administering TGF-β to a mammal. This is because, as set forth previously elsewhere herein, contacting a cell with TGF-β increases REMODELIN expression and increased REMODELIN expression can provide therapeutic benefit to the mammal, including, but not limited to, increased bone formation, accelerated wound healing, and the like. Thus, the skilled artisan would understand, based upon the disclosure provided herein, that the present invention includes increasing the level of REMODELIN expression by administering TGF-β to a mammal.

The invention includes a method of affecting cellular gene expression by administering an isolated nucleic acid encoding REMODELIN or an isolated nucleic acid antisense to a nucleic acid encoding REMODELIN. This is because, as the data presented herein demonstrate, expression of a nucleic acid encoding REMODELIN or expression of a nucleic acid antisense to a nucleic acid encoding REMODELIN affects the level of several cellular genes including, but not limited to, TGF-β1, collagen IIIα1, ostiopontin, biglycan, alkaline phosphatase (ALP), and bone morphogenic protein 4 (BMP-4). Thus, increasing the level of REMODELIN in a cell, such as by, among other things, administering a nucleic acid encoding REMODELIN to the cell and expressing the REMODELIN therefrom under the control of a promoter

that drives increased level of expression, can be used to control the expression of various proteins, including, but not limited to, TGF-β1, collagen IIIα1, ostiopontin, biglycan, ALP and BMP-4, where affecting the expression of these proteins provides a benefit to a mammal.

The data disclosed herein demonstrate that REMODELIN can inhibit expression of bone differentiation markers thereby inhibiting calcification/ossification. Thus, the skilled artisan would appreciate, based upon the disclosure provided herein, that REMODELIN, by inhibiting bone differentiation markers, can inhibit premature calcification/ossification. Therefore, the present invention encompasses a method of inhibiting calcification comprising increasing expression of REMODELIN in a cell. This is because the data disclosed herein demonstrate that expression of REMODELIN inhibits bone differentiation markers thereby inhibiting calcification.

In addition to replacing defective cells with repaired cells or normal cells from syngeneic, immunologically-matched donors, the method of the invention may also be used to facilitate expression of a desired protein that when secreted in the an animal, has a beneficial effect. That is, cells may be isolated, furnished with a gene encoding REMODELIN and introduced into the donor or into a syngeneic matched recipient wherein expression of exogenous REMODELIN exerts a therapeutic effect.

One skilled in the art would understand, based upon the disclosure provided herein, that secretion of REMODELIN from a cell is contemplated in the present invention. That is, the routineer would appreciated, based upon the disclosure provided herein, that secretion of REMODELIN from a cell can be a useful therapeutic method and that the present invention includes secretion of REMODELIN from a cell. Secretion of REMODELIN from a cell can be effected according to standard methods well-known in the art and methods to be developed in the future. Such methods include, but are not limited to, covalently linking a nucleic acid encoding a signal peptide of a secreted molecule (*e.g.*, insulin; MALLVHFLPLLALLALWEPKPTQA [SEQ ID NO:8]) to the 5' end of an isolated nucleic acid encoding REMODELIN. A wide plethora of signal sequences that can be used to mediate secretion of a protein from a cell are available and well-known in the art and the invention includes those as

well as sequences to be developed in the future to drive secretion of a protein from a cell.

This aspect of the invention relates to gene therapy in which therapeutic amounts of REMODELIN are administered to an individual. That is, according to some aspects of the present invention, recombinant cells transfected with either nucleic acid encoding REMODELIN, antisense nucleic acids, or a knock-out targeting vector of the invention, can be used as cell therapeutics to treat a disease, disorder or a condition characterized by altered expression of REMODELIN, including the lack of expression of REMODELIN.

In particular, a gene construct that comprises a heterologous gene which encodes REMODELIN is introduced into cells. These recombinant cells are used to purify isolated REMODELIN, which was is administered to an animal. One skilled in the art would understand, based upon the disclosure provided herein, that instead of administering an isolated REMODELIN polypeptide, REMODELIN can be administered to a mammal in need thereof by administering to the mammal the recombinant cells themselves. This will benefit the recipient individual who will benefit when the protein is expressed and secreted by the recombinant cell into the recipient's system.

According to the present invention, gene constructs comprising nucleotide sequences of the invention are introduced into cells. That is, the cells, referred to herein as "recombinant cells," are genetically altered to introduce a nucleic acid encoding REMODELIN or a nucleic acid that inhibits REMODELIN expression in and/or secretion by the recombinant cell (*e.g.*, an antisense REMODELIN nucleic acid, a nucleic acid encoding an anti-REMODELIN antibody, TGF-β receptor, and the like), thereby mediating a beneficial effect on an recipient to which the recombinant cell is administered. According to some aspects of the invention, cells obtained from the same individual to be treated or from another individual, or from a non-human animal, can be genetically altered to replace a defective REMODELIN gene and/or to introduce a REMODELIN gene whose expression has a beneficial effect on the individual, or to inhibit REMODELIN expression which can have a beneficial effect on the individual.

In some aspects of the invention, an individual suffering from a disease, disorder or a condition can be treated by supplementing, augmenting and/or replacing defective or deficient nucleic acid encoding REMODELIN by providing an isolated recombinant cells containing gene constructs that include normal, functioning copies of a nucleic acid encoding REMODELIN. This aspect of the invention relates to gene therapy in which the individual is provided with a nucleic encoding REMODELIN for which they are deficient in presence and/or function. The isolated nucleic acid encoding REMODELIN provided by the cell compensates for the defective REMODELIN expression of the individual, because, when the nucleic acid is expressed in the individual, a protein is produced which serves to alleviate or otherwise treat the disease, disorder or condition in the individual. Such nucleic acid preferably encodes a REMODELIN polypeptide that is secreted from the recombinant cell.

In all cases in which a gene construct encoding REMODELIN is transfected into a cell, the nucleic acid is operably linked to an appropriate promoter/regulatory sequence which is required to achieve expression of the nucleic acid in the recombinant cell. Such promoter/regulatory sequences include but are not limited to, constitutive and inducible and/or tissue specific and differentiation specific promoters, and are discussed elsewhere herein. Constitutive promoters include, but are not limited to, the cytomegalovirus immediate early promoter and the Rous sarcoma virus promoter. In addition, housekeeping promoters such as those which regulate expression of housekeeping genes may also be used. Other promoters include those which are preferentially expressed in cells of the central nervous system, such as, but not limited the promoter for the gene encoding glial fibrillary acidic protein. In addition, promoter/regulatory elements may be selected such that gene expression is inducible. For example, a tetracycline inducible promoter may be used (Freundlich et al., 1997, Meth. Enzymol. 283:159-173).

The gene construct is preferably provided as an expression vector which includes the coding sequence of a mammalian REMODELIN of the invention operably linked to essential promoter/regulatory sequences such that when the vector is transfected into the cell, the coding sequence is expressed by the cell. The coding sequence is operably linked to the promoter/regulatory elements necessary for

expression of the sequence in the cells. The nucleotide sequence that encodes the protein may be cDNA, genomic DNA, synthesized DNA or a hybrid thereof or an RNA molecule such as mRNA.

The gene construct, which includes the nucleotide sequence encoding REMODELIN operably linked to the promoter/regulatory elements, may remain present in the cell as a functioning episomal molecule or it may integrate into the chromosomal DNA of the cell. Genetic material may be introduced into cells where it remains as separate genetic material in the form of a plasmid. Alternatively, linear DNA which can integrate into a host cell chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be introduced into the cell.

In order for genetic material in an expression vector to be expressed, the promoter/regulatory elements must be operably linked to the nucleotide sequence that encodes the protein. In order to maximize protein production, promoter/regulatory sequences may be selected which are well suited for gene expression in the desired cells. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce recombinant genetic material as expression vectors which are functional in the desired cells.

It is also contemplated that promoter/regulatory elements may be selected to facilitate tissue specific expression of the protein. Thus, for example, specific promoter/regulatory sequences may be provided such that the heterologous gene will only be expressed in the tissue where the recombinant cells are implanted. Additionally, the skilled artisan would appreciate, based upon the disclosure provided herein, that the REMODELIN promoter can be operably linked to a nucleic acid of interest thereby directing the expression of the nucleic acid at the site of tissue or organ injury and wounding. More specifically, the REMODELIN promoter can be used, but is not limited, to direct expression of an angiogenic growth factor to promote angiogenesis after myocardial infarction. Similarly, the REMODELIN promoter can drive expression of a nucleic acid of interest where such expression is beneficial where

tissue ischemia and impaired wound healing are a problem (*e.g.*, ulcerations of the skin, and the like).

One skilled in the art would understand, based upon the disclosure provided herein, that the preferred tissues where the expression or lack of expression of REMODELIN is to be targeted include, but are not limited to, ulcerations of the skin, bone fractures, and the like. In addition, promoter/regulatory elements may be selected such that gene expression is inducible. For example, a tetracycline inducible promoter may be used (Freundlich et al., 1997, Meth. Enzymol. 283:159-173).

Without wishing to be bound by any particular theory, the nucleic acid encoding REMODELIN preferably includes a putative signal sequence as disclosed elsewhere herein (*e.g.*, amino acids 1 to 32 of human REMODELIN; SEQ ID NO:3) and amino acids 1 to 32 of rat REMODELIN$_S$ (SEQ ID NO:1), which may direct the transport and secretion of the REMODELIN encoded by the isolated nucleic acid in the recombinant cell. The signal sequence is likely processed and removed upon secretion of the mature REMODELIN protein from the cell. Alternatively, without wishing to be bound by any particular theory, the putative signal sequence may not be cleaved, but may instead be a transmembrane domain.

In addition to providing cells with recombinant genetic material that either corrects a genetic defect in the cells, that encodes a protein which is otherwise not present in sufficient quantities and/or functional condition so that the genetic material corrects a genetic defect in the individual, and/or that encodes a protein which is useful as beneficial in the treatment or prevention of a particular disease, disorder or condition associated therewith, and that inhibits expression of REMODELIN in the cell (*e.g.*, a knock-out targeting vector, an antisense nucleic acid, and the like), genetic material can also be introduced into the recombinant cells used in the present invention to provide a means for selectively terminating such cells should such termination become desirable. Such means for targeting recombinant cells for destruction may be introduced into recombinant cells.

According to the invention, recombinant cells can be furnished with genetic material which renders them specifically susceptible to destruction. For example, recombinant cells may be provided with a gene that encodes a receptor that

can be specifically targeted with a cytotoxic agent. An expressible form of a gene that can be used to induce selective cell death can be introduced into the recombinant cells. In such a system, cells expressing the protein encoded by the gene are susceptible to targeted killing under specific conditions or in, the presence or absence of specific agents. For example, an expressible form of a herpes virus thymidine kinase (herpes tk) gene can be introduced into the recombinant cells and used to induce selective cell death. When the introduced genetic material that includes the herpes tk gene is introduced into the individual, herpes tk will be produced. If it is desirable or necessary to kill the implanted recombinant cells, the drug gangcyclovir can be administered to the individual which will cause the selective killing of any cell producing herpes tk. Thus, a system can be provided which allows for the selective destruction of implanted recombinant cells.

One skilled in the art would understand, based upon the disclosure provided herein, that the present invention encompasses production of recombinant cells to either provide REMODELIN to or inhibit REMODELIN expression in a mammal. That is, the cells can be used to administer REMODELIN to an animal or to deliver a molecule (*e.g.*, a knock-out targeting vector, an antisense nucleic acid, a ribozyme, and antibody that specifically binds with REMODELIN, and the like).

Administration of REMODELIN to an animal can be used as a model system to study the mechanism of action of REMODELIN or to develop model systems useful for the development of diagnostics and/or therapeutics for diseases, disorders or conditions associated with REMODELIN expression.

Further, the delivery of REMODELIN to an animal mediated by administration of recombinant cells expressing and secreting REMODELIN can also be used to treat or alleviate a disease, disorder or condition where increasing the level of REMODELIN mediates a therapeutic effect. More specifically, administration of REMODELIN to an animal by administering a recombinant cell expressing a nucleic acid encoding REMODELIN can be useful for treatment of impaired wound healing, bone fracture, and impaired bone formation, among other things.

Alternatively, administration of recombinant cells comprising a nucleic acid the expression of which inhibits or reduces REMODELIN expression, activity,

and/or secretion from a cell, can be used as a model for the development of diagnostics and/or therapeutics useful for diseases, disorders or conditions associated with or mediated by REMODELIN expression, activity, and/or secretion. The present invention encompasses that the recombinant cells can produce the molecule that inhibits REMODELIN expression thereby providing such molecule to the animal. Alternatively, without wishing to be bound by any particular theory, the recombinant cells themselves, which are otherwise functional cells, except for the inability to express REMODELIN, can perform the functions of otherwise identical but non-recombinant cells, without being subject to the REMODELIN signaling pathway.

Cells, both obtained from an animal, from established cell lines that are commercially available or to be developed, or primary cells cultured *in vitro*, can be transfected using well known techniques readily available to those having ordinary skill in the art. Thus, the present invention is not limited to obtaining cells from a donor animal or from the patient animal itself. Rather, the invention includes using any cell that can be engineered using a nucleic acid of the invention such that the recombinant cell either expresses REMODELIN (where it did not express REMODELIN prior to being engineered, or where the cell produced REMODELIN at a different level prior to the introduction of the nucleic acid into the cell) or the recombinant cell does not express REMODELIN or expresses it at a lower level (where it expressed REMODELIN before or expressed REMODELIN at a different level prior to introduction of the nucleic acid into the cell).

Nucleic acids can be introduced into the cells using standard methods which are employed for introducing a gene construct into cells which express the protein encoded by the gene or which express a molecule that inhibits REMODELIN expression. In some embodiments, cells are transfected by calcium phosphate precipitation transfection, DEAE dextran transfection, electroporation, microinjection, liposome-mediated transfer, chemical-mediated transfer, ligand mediated transfer or recombinant viral vector transfer.

In some embodiments, recombinant adenovirus vectors are used to introduce DNA having a desired sequence into the cell. In some embodiments, recombinant retrovirus vectors are used to introduce DNA having a desired sequence

into the cell. In some embodiments, standard calcium phosphate, DEAE dextran or lipid carrier mediated transfection techniques are employed to incorporate a desired DNA into dividing cells. Standard antibiotic resistance selection techniques can be used to identify and select transfected cells. In some embodiments, DNA is introduced directly into cells by microinjection. Similarly, well known electroporation or particle bombardment techniques can be used to introduce foreign DNA into cells. A second gene is usually co-transfected with and/or covalently linked to the nucleic acid encoding REMODELIN, or knock-out targeting vector or antisense molecule thereto. The second gene is frequently a selectable antibiotic-resistance gene. Transfected recombinant cells can be selected by growing the cells in an antibiotic that kills cells that do not take up the selectable gene. In most cases where the two genes are unlinked and co-transfected, the cells that survive the antibiotic treatment contain and express both genes.

Where an isolated REMODELIN polypeptide, an antibody that specifically binds with REMODELIN, a REMODELIN antisense nucleic acid, TGF-β1, TGF-β1 receptor, and/or recombinant cells of the invention are administered to an animal either to increase or reduce the level of REMODELIN present in the animal, one skilled in the art would understand, based upon the disclosure provided herein, that the amount of the polypeptide, nucleic acid, antibody, TGF-β1, TGF-β1 receptor, or cell to be administered to the animal can be titrated by assessing the level of expression of REMODELIN or the level of REMODELIN polypeptide or nucleic acid encoding REMODELIN present in the tissues of the animal.

Methods for assessing the level of REMODELIN (*e.g.*, using anti-REMODELIN antibodies in Western blot or other immune-based analyses such as ELISA) and/or methods for assessing the level of REMODELIN expression in a cell and/or tissues (*e.g.*, using Northern blot analysis, RT-PCR analysis, *in situ* hybridization, and the like) are disclosed herein or are well known to those skilled in the art. Such assays can be used to determine the "effective amount" of REMODELIN (whether using an isolated nucleic acid, antibody, antisense nucleic acid, ribozyme, recombinant cell, and the like) to be administered to the animal in order to reduce or increase the level of REMODELIN to a desired level.

C. Methods of diagnosis and assessment of therapies

The present invention includes methods of diagnosis certain diseases, disorders, or conditions such as, but not limited to, negative remodeling, arterial restenosis, adventitial fibrosis, excessive wound healing responses, scarring, keloids, excessive bone formation, fracture healing, ectopic ossification (malignant and benign), fibrosis in any organ or tissue (*e.g.*, liver fibrosis and lung fibrosis), altered bone density, altered bone growth, collagen diseases, and the like, which are associated with or mediated by abnormal expression of REMODELIN.

The invention includes a method of diagnosing tissue damage, negative remodeling, arterial restenosis, adventitial fibrosis, excessive wound healing responses, scarring, keloids, excessive bone formation, fracture healing, ectopic ossification (malignant and benign), fibrosis in any organ or tissue (*e.g.*, liver fibrosis and lung fibrosis), altered bone density, altered bone growth, premature calcification, collagen-related diseases, and the like, in a patient mammal. This is because, as demonstrated by the data disclosed herein, there is a correlation between altered expression of REMODELIN, when compared to expression of REMODELIN in otherwise identical but undamaged, normal tissue, and tissue injury, negative remodeling, arterial restenosis, adventitial fibrosis, excessive wound healing responses, scarring, keloids, excessive bone formation, fracture healing, ectopic ossification (malignant and benign), fibrosis in any organ or tissue (*e.g.*, liver fibrosis and lung fibrosis), altered bone density, altered bone growth, premature calcification, collagen diseases, and the like, such that assessing the level of REMODELIN expression is a useful diagnostic for these diseases, disorders, or conditions associated with altered expression of REMODELIN.

The method comprises obtaining a biological sample from the mammal and comparing the level of REMODELIN (expression, amount, activity) in the sample with the level of REMODELIN in a sample from a normal person who is not afflicted with tissue damage, ectopic ossification, and organ fibrosis. A higher level of REMODELIN in the sample from the patient compared with the level of REMODELIN in the sample obtained from a person not afflicted with tissue damage,

ectopic ossification, and organ fibrosis an indication that the patient is afflicted with tissue damage, ectopic ossification, and organ fibrosis. This is because, as disclosed elsewhere herein, an increased level of REMODELIN expression is associated with tissue damage, ectopic ossification, organ fibrosis, bone mineralization, skin wounding, bone density and/or bone growth, lack of dorsal closure, *spina bifida*-like phenotype, collagen-related phenotypes, and vascular injury.

In one aspect, the biological sample is selected from the group consisting of a lung biopsy, an aorta sample, a smooth muscle cell (SMC) sample, an endarterectomy sample, a liver biopsy, any biopsy from a wound, and the like.

The invention includes a method of assessing the effectiveness of a treatment for arterial restenosis in a mammal. The method comprises assessing the level of REMODELIN expression, amount, and/or activity, before, during and after a specified course of treatment for arterial stenosis since arterial restenosis and/or arterial fibrosis is associated with increased REMODELIN expression. This is because, as stated previously elsewhere and demonstrated by the data disclosed herein, REMODELIN expression, amount and/or activity is associated with or mediates decreased increased cell proliferation which is feature of certain disease states (*e.g.*, negative remodeling, adventitial fibrosis and arterial restenosis). Thus, assessing the effect of a course of treatment upon REMODELIN expression/amount/activity indicates the efficacy of the treatment such that a lower level of REMODELIN expression, amount, or activity indicates that the treatment method is successful.

The data disclosed herein should allow the identification and characterization of the REMODELIN ligand/receptor. This is useful since antagonism of the REMODELIN ligand, receptor, or both, should provide useful in treatment of diseases, disorders or conditions mediated by REMODELIN ligand/receptor signaling such as, but not limited to, arterial restenosis, negative remodeling, adventitial fibrosis, fibrosis in any organ or tissue (*e.g.*, liver, lung, among others), hypertrophic scar tissue (*i.e.*, keloids), excessive bone formation, ectopic ossification (malignant and benign), and the like.

IX. Kits

The invention includes various kits which comprise a compound, such as a nucleic acid encoding REMODELIN, an antibody that specifically binds REMODELIN, a nucleic acid complementary to a nucleic acid encoding REMODELIN but in an antisense orientation with respect to transcription, and/or compositions of the invention, an applicator, and instructional materials which describe use of the compound to perform the methods of the invention. Although exemplary kits are described below, the contents of other useful kits will be apparent to the skilled artisan in light of the present disclosure. Each of these kits is included within the invention.

In one aspect, the invention includes a kit for alleviating a disease mediated by altered expression of REMODELIN. The kit is used pursuant to the methods disclosed in the invention. Briefly, the kit may be used to contact a cell with a nucleic acid complementary to a nucleic acid encoding REMODELIN where the nucleic acid is in an antisense orientation with respect to transcription to reduce expression of REMODELIN, or with an antibody that specifically binds with REMODELIN, wherein the decreased expression, amount, or activity of REMODELIN mediates an beneficial effect.

The kit further comprises an applicator useful for introducing the nucleic acid or antibody protein of the invention into the cell. The particular applicator included in the kit will depend on, *e.g.*, the recombinant DNA method used to introduce the nucleic acid into the cell and such applicators are well-known in the art and may include, among other things, a pipette, a syringe, a dropper, and the like. Moreover, the kit comprises an instructional material for the use of the kit. These instructions simply embody the disclosure provided herein.

The kit includes a pharmaceutically-acceptable carrier. The composition is provided in an appropriate amount as set forth elsewhere herein. Further, the route of administration and the frequency of administration are as previously set forth elsewhere herein.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

EXAMPLES

The experiments presented in this example may be summarized as follows.

In order to identify novel factors involved in mediating arterial remodeling in response to injury, suppressive subtractive hybridization was performed using mRNA from normal and balloon-injured rat arteries. A novel nucleic acid sequence was identified using this approach and a full length 1235 bp cDNA clone was isolated by screening a cDNA library prepared from 8 day balloon-injured rat carotid arteries and aortae. This cDNA clone comprises an open reading frame (ORF) of about 245 amino acids having no significant homology to any known protein. This protein, referred to herein as REMODELIN, was previously termed REMODEL, and more previously termed AIBE for Adventitia Inducible and Bone Expressed protein. REMODELIN comprises, *inter alia*, a potential transmembrane domain and five potential N-myristoylation sites which can target the molecule to the cell membrane.

In situ hybridization analysis disclosed that REMODELIN mRNA expression is remarkably restricted to the adventitia of balloon-injured vessels with maximal expression detected at 8 days after carotid artery balloon denudation and with no detectable expression in normal arteries. REMODELIN expression in the adventitia was no longer detectable at 6 weeks after balloon injury.

The data disclosed herein further demonstrate that during mouse embryogenesis, REMODELIN expression was prominent in developing bone starting at about 12 days post coitus (dpc), while Northern blot analysis demonstrated that only

low levels of REMODELIN expression were detected in brain and lung of the adult animal.

Antibodies specific for REMODELIN were generated and used to characterize expression of the protein by in vitro translation, in various cell lines which endogenously express REMODELIN, injured arteries, and cells transfected with a REMODELIN expression vector.

REMODELIN was shown to alter the expression of several cellular genes including TGF-β1, collagen III, biglycan, osteopontin, ALP, and BMP-4. Further, REMODELIN was shown to inhibit *Cbfa1*-mediated activation of the osteocalcin promoter, as determined using a luciferase assay.

Injection of REMODELIN mRNA into frog embryos caused severe developmental abnormalities, including, but not limited to, inhibition of FGF-induced mesoderm formation, failure of neural tissue cells to migrate, and failure of dorsal closure, abnormal head development, and formation of a split tail.

MC3T3-E1 cells transfected with REMODELIN antisense exhibited elongated, fibroblastic morphology, and exhibited increased cell turnover, suggesting that REMODELIN is involved in cell-matrix and cell-cell interaction(s).

Analysis of REMODELIN transgenic mice revealed severe phenotypes including hemorrhaging, dwarfism, skeletal abnormalities including decreased bone density, and severe myopathy.

The Results of the experiments presented in this example are now described.

Identification of injury inducible factor

Suppressive subtractive hybridization was performed between cDNA expressed in normal rat carotid artery/aorta and cDNA expressed in 8 day balloon injured carotid/aorta using the PCR-Select kit from Clontech Laboratories, Inc. (Palo Alto, CA), to identify genes that are involved in the arterial remodeling response to injury. The normal vessel provided the "driver" cDNA and the injured vessel provided the "tester" cDNA.

Partial sequences of approximately 300 clones were obtained by automated sequencing and the sequence identities were determined by searching GenBank databases, including non-redundant and EST (expressed sequence tag) databases which are publicly available at http://www.ncbi.nlm.nih.gov/blast/blast.cgi. Those sequences not matching a known gene (usually corresponding murine or human ESTs) were identified in the database and were pursued further.

Duplicate slot blots containing the series of cDNA clones were hybridized with ^{32}P-dCTP labeled cDNA prepared from either normal vessel RNA or from balloon-injured vessel RNA. The clones that exhibited increased expression in the injured vessels were then further tested using Northern blot technique with RNA from both normal and balloon-injured rat arteries.

The data disclosed herein demonstrate that REMODELIN expression was essentially not detectable in normal vessels while injured vessels exhibited a dominant 1.2 kb transcript (Figure 1B-1). The sequences exhibiting detectable increased expression in the injured vessels compared with normal vessels were further examined for expression in various organs using Northern blot. In order to select for genes that might be specific for the vasculature, those clones that were predominantly expressed in vascular tissues like lung and brain, in addition to showing expression in the aorta or carotid artery, were pursued. The data disclosed herein demonstrate that REMODELIN showed low levels of expression in lung and brain (Figure 1A-1).

Clones identified using the above-described screening approach were then used to make ^{35}S-UTP labeled sense and antisense strand which were, in turn, used for *in situ* hybridization on normal and balloon–injured rat carotid artery sections (4, 8, 14, and 28 days after injury), as well as on sections from staged mouse embryos.

To determine expression in quiescent versus proliferating/migrating endothelium, *en face* preparations of 7 and 14 day injured aortas were also used in the *in situ* hybridization study as described in Lindner and Reidy (1993, Circ. Res. 73:589-595). In the injured aortae, endothelial regeneration occurs from the intercostal arteries giving rise to migrating/proliferating endothelial cells at the wound edge as well as quiescent endothelium in the monolayer away from the wound edge.

Among the clones exhibiting modulated expression in response to injury, REMODELIN was expressed in the adventitia of injured vessels but was absent from normal adventitia (Figures 2A and 2B). Strong expression of REMODELIN was detected at 8 days after injury with less expression at 14 days (Figures 2B and 2C). Surprisingly, there was no appreciable REMODELIN expression detected in the media and in the developing neointima despite the fact that Smooth Muscle Cells (SMC) *in vitro* expressed the 1.2 kb transcript (Figures 2B and 2C). No other gene is known in the art which is specifically induced in the adventitia and is not detectably induced in the neointima. Longer exposure or loading of larger amounts of RNA from *in vitro* SMC also revealed a less abundant transcript of about 3.5 kb (Figure 1A-1).

At 4 weeks after balloon injury, REMODELIN expression was nearly undetectable in the adventitia. At the time when REMODELIN is expressed, the adventitia shows rapid proliferation of myofibroblasts as well as a subsequent sharp decline in cell number that is accompanied by abundant synthesis of collagens type I and type III (Smith et al., 1999, Cir. Res. 84:1212-1222). Interestingly, in the staged mouse embryos, REMODELIN expression was detected in the mesoderm at 11.5 days post coitus (dpc) (Figure 2E), which expression later became restricted to the developing bone (Figure 2F). Lower levels of REMODELIN expression were also detected in the cortical bone of a femur from a rat pup. During development expression of REMODELIN was prominent in developing bone such as the skull (Figures 2F, 2G and 2H). REMODELIN continued to be expressed in osteoblasts adjacent to mineralized bone (Figures 2I and 2J).

In addition, full thickness skin incisions undergoing wound healing and remodeling revealed strong expression along the incision (Figure 2L) while no expression was detectable in normal skin (Fig. 2K).

A full length REMODELIN clone was obtained using a size-fractionated (500 bp cut-off) cDNA library prepared using mRNA extracted from 8 day balloon-injured rat aortas and carotid arteries using a Lambda Zap Express system (Stratagene, La Jolla, CA). After excision with a helper phage, the isolated cDNA clones were ligated into the pBK-CMV vector (Stratagene, La Jolla, CA), which allows for convenient expression in mammalian cells using the CMV promoter. This library

is expected to contain sequences expressed in proliferating SMC, endothelial cells (EC), and fibroblasts as well as their quiescent counterparts. In addition, sequences from inflammatory cells, predominantly macrophages, are also expected to be present in the library.

The 230 bp REMODELIN clone obtained using the differential screen approach was then used to probe the library and six clones were isolated and sequenced. Five clones started within 50 bp upstream of a putative translational AUG start site at nucleotide position 116 (Figure 4Ai). The longest clone contained an additional 60 bp of 5' sequence which contained a potential additional in-frame AUG translational start site at position 19 (Figure 4Ai). The 230 bp clone was designated REMODELIN-short (REMODELIN$_S$) and the 290 bp REMODELIN clone was designated REMODELIN-long (REMODELIN$_L$).

Searching The Institute for Genomic Research (TIGR) sequence database for the human homolog of REMODELIN, identified a 771 bp of 3' sequence. Using RNA from cultured human aortic SMC, 5' RACE (rapid amplification of cDNA ends) cloning was performed to identify the missing approximately 500 bp sequence located at the 5' end. The sequence alignment of rat (SEQ ID NO:2) and human REMODELIN (SEQ ID NO:4) is shown in Figure 4. Interestingly, the human sequence did not have the additional 5' AUG translational start site but only contained the AUG codon at position 114. A 5' primer located upstream of this AUG start site and a 3' primer were designed to verify that the AUG start site at position 19 of the rat sequence was not a cloning artifact. Reverse transcription polymerase chain reaction (RT-PCR) analysis and sequencing was performed to confirm the presence of the AUG-19 codon. The overall identity between the human and rat REMODELIN sequence was 78.3% at the nucleotide level using a blast 2 algorithm search as provided at the web site http://www.ncbi.nlm.nih.gov/gorf/bl2.html, which web site is publicly available. Further, homology between the rat (SEQ ID NO:2) and human REMODELIN (SEQ ID NO:4) amino acid sequences is greater than about 97% using the blast 2 algorithm search strategy described previously.

Amino acid sequence and domains within REMODELIN

In vitro translation was performed using a kit (Promega Corp., Madison, WI) comprising rabbit reticulocyte lysate and ^{35}S-methionine. The data disclosed herein demonstrate that REMODELIN$_L$ contained two putative transcriptional start sites compatible with the Kozak rule (*i.e.*, at positions 19 and 116) while REMODELIN$_S$ had only the AUG$_{116}$ site. *In vitro* translation revealed that the REMODELIN$_L$ construct expressed preferentially the long form but, to a lesser extent, the short form of REMODELIN was also expressed from the REMODELIN$_L$ construct (Figure 5A). However, the REMODELIN$_S$ construct expressed only the shorter form. The apparent molecular weights of the long and short form were approximately 34 kDa and 30 kDa, respectively (Figure 5A). Since the human sequence does not have the additional AUG codon at the 5' end, it is most likely that *in vivo* translation starts at AUG$_{116}$. This is predicted to result in a 245 amino acid (aa) rat protein while the human homolog has about 243 amino acids (SEQ ID NO:4) due to a 2 amino acid deletion in the amino terminus.

A leucine-rich hydrophobic region is located near the amino terminus and, without wishing to be bound by any particular theory, this region is predicted to be a cleavable signal peptide (from about amino acid residue number 1 to amino acid residue 32), which would result in a 213 aa mature peptide having a molecular weight of about 23.1 kDa and a theoretical pI of 6.57 for the human REMODELIN. However, if the aa1-aa32 peptide is not a signal sequence and does not get cleaved, it is predicted to be a definitive transmembrane domain. The rat REMODELIN$_L$ protein would result in a 277 aa protein which lacks a predicted signal sequence (SEQ ID NO:2).

At the amino acid level, human and rat REMODELIN were 95% identical (Figure 4B). The carboxy terminal half of the molecule was more highly conserved with over 99% identity between the two species. Remarkably, the amino acid composition contains 4.7% cysteine, 2.4% tyrosine, and 2% tryptophan residues. A glycine-rich domain also found in many collagens is located between amino acid residue 59 and amino acid residue 93. A putative N-glycosylation site is located at about amino acid residue 188 to about amino acid residue 191, a putative protein kinase C (PKC) phosphorylation site at amino acid residue 146 to amino acid residue

148, and four casein kinase II (CD2) phosphorylation sites. Furthermore, there are 5 N-myristoylation sites located throughout the molecule. A summary of these putative motifs is depicted in Figure 3.

Functional characterization of REMODELIN

Contrary to SMC in the vessel wall, cultured rat aortic SMC growing in DMEM supplemented with 10% bovine serum expressed REMODELIN mRNA. Expression was inducible by TGF-β in 3T3 cells as well as SMC with peak expression detected after 8 hours of stimulation. Expression levels were still elevated after 24 hours and no induction followed stimulation of cells with FGF-2.

Since REMODELIN is also expressed in bone, expression in MC3T3 cells, a bone derived cell line, was also examined. The response to TGF-β stimulation was similar to SMC and NIH3T3 cells with maximal induction occurring after 8 hours (Figure 1C). Stimulation with bone morphogenetic protein-4 (BMP-4) caused a similar induction of REMODELIN mRNA in MC3T3 cells as TGF-β (Figure 1C).

Antibodies that specifically bind REMODELIN

The peptide corresponding to the carboxy terminal 15 amino acids of REMODELIN was coupled to keyhole limpet hemocyanin (KLH) and used to immunize two rabbits. One of the rabbits produced antiserum that had a titer of greater than about 1:64,000. The antiserum (used at a 1:5000 dilution) detected a specific band with a molecular weight of approximately 30 kDa in lysates prepared from 10 day balloon-injured rat carotid arteries while this band was absent in lysates prepared from normal vessels (Figure 5C). The same specific band was also detected in lysates from cultured SMC.

In addition, SMC cultured *in vitro* also expressed a slightly larger immunoreactive band of approximately 34 kDa. However, concentrated conditioned medium obtained from SMC contained no detectable immunoreactivity, indicating that if any REMODELIN is secreted, it is at low levels beyond the level of detection. Further, the detectable bands were specific since the preimmune IgG from the same rabbit did not react with these protein bands (Figure 5C).

Expression of GFP-tagged REMODELIN in transfected cells

REMODELIN cDNA was cloned into a green fluorescent protein (GFP)- and hemagglutinin (HA)- tagged expression vector to produce REMODELIN/tag polypeptide fusion proteins. The localization of the GFP-REMODELIN fusion protein was then assessed with regard to its cellular localization following transfection of NIH3T3 and 293 cells with the construct encoding the fusion protein. The data disclosed herein demonstrate that fluorescence was distributed homogeneously throughout the cell with absence of GFP in the nucleus. This staining pattern is compatible with cytosolic and/or cell membrane localization. The transfection efficiency obtained using the GFP-REMODELIN construct was consistently lower than with the GFP control vector.

Expression of myc-tagged REMODELIN in transfected cells

The coding region of rat REMODELIN was cloned into a mammalian expression vector comprising a myc tag at the carboxy terminus (pcDNA3.1myc/his, Invitrogen, Carlsbad, CA). The sequence of the coding region used in the construct is depicted in Figure 10 (SEQ ID NO:9).

Transient transfections were performed using bovine aortic endothelial cells (BAE) and NIH3T3 cells. The cell lines were analyzed for transgene expression 24 hours and 48 hours after transfection using Western blotting and immunostaining using an anti-myc monoclonal antibody (Zymed, South San Francisco, CA).

Interestingly, the data disclosed herein demonstrate that very little expression remained at 48 hours post-transfection (Figure 5F). Without wishing to be bound by any particular theory, these data suggest that the transfected cells were lost from the culture. In comparison, the same cell line transfected in parallel with an unrelated cDNA (EP1) using the same vector exhibited significantly higher levels of expression at 48 hours than at 24 hours (Figure 5F).

The possibility that overexpression of REMODELIN results in cell death was examined further using immunohistochemistry, confocal microscopy and flow cytometry for cell cycle analysis. BAE cells transiently transfected with myc-

tagged REMODELIN exhibited a 15% increase in accumulation of cells in G0-G1 at 24 hours post-transfection (transfection efficiency approximately 15%). Western blotting of these transfectants using the anti-myc antibody demonstrated that by 48 hours post-transfection very little immunoreactivity remained, indicating that the transfected cells were lost from the culture.

Confocal microscopy of REMODELIN transfected NIH3T3 cells demonstrated localization of the myc-tagged protein in very small vesicles distributed throughout the cytoplasm (Figure 5B).

Expression of REMODELIN in various cell types

Vessel wall lysates were prepared from rat carotid arteries harvested at 1, 4, 7, 14, and 28 days after balloon injury. These lysates (30 micrograms of protein in each lane) were analyzed by immunoblotting using an antibody raised against the carboxyterminal peptide of REMODELIN. The highest levels of REMODELIN were seen at 4 and 7 days after injury with a decline to near control levels at 28 days post injury (Figure 5C).

Smooth muscle cells in vitro expressed REMODELIN mRNA while this cell type showed very little expression in vivo. Therefore expression of REMODELIN was examined using a variety of different cell lines using immunoblotting analysis (Figure 5D). The cell lines included NIH3T3, bovine aortic (BAE), PAC-1 (a rat smooth muscle cell line), A7r5 (a rat smooth muscle cell line), RASMC (rat aortic SMC primary culture), 293, BASMC (bovine aortic SMC), 10T1/2, human umbilical vein endothelial cells (HUVEC), A431, and human aortic SMC (HASMC). The data disclosed herein demonstrate detection of a prominent immunoreactive band with an apparent molecular weight of about 34k Da that was present in all cell lines tested. These data indicate that the antibody reacted with mouse, rat, bovine, and human homologs of REMODELIN (Figure 5D). A less abundant protein band having an apparent molecular weight of about 30 kDa was present in some cell lines and some additional larger immunoreactive bands were also detected. Without wishing to be bound by any particular theory, the 30 kDa band may reflect differences in glycosylation or phosphorylation of REMODELIN.

Regulation of REMODELIN expression

The regulation of REMODELIN expression by TGF-ß was further investigated using MC3T3 cells, a bone derived cell line. Immunoblotting of cell lysates harvested after 24, 48, and 72 hours after stimulation with TGF-ß demonstrated increased expression levels of REMODELIN protein compared to controls (Figure 5E).

The data disclosed herein demonstrate that blocking signaling via the TGF-ß receptor type II by the addition of a soluble TGF-ß receptor type II (Biogen, Cambridge, MA) to the cells, inhibited expression of REMODELIN protein (Figure 5E). MC3T3 cells were treated with 1ng/ml of TGF-ß1 (TGF-ß) or 100ng/ml soluble TGF-ß receptor type II (sol. TGF-ßRII), the cells were harvested at various time points, and 30 micrograms of protein were applied to each polyacrylamide gel lane (Figure 5E). The data disclosed herein demonstrate that TGF-ß1 stimulated REMODELIN expression while inhibition of TGF-ß signaling inhibited REMODELIN expression.

Effects of REMODELIN on gene expression

Stable NIH3T3 and MC3T3 cell lines overexpressing REMODELIN under the control of the CMV promoter were established. To identify genes whose expression might be changed in response to altered levels of REMODELIN, Northern blots from REMODELIN overexpressing and antisense REMODELIN transfected cell lines were performed (Figure 6). REMODELIN overexpressing NIH3T3 cells expressed considerable lower levels of TGF-β1, collagen III, and biglycan while the mRNA levels of the same genes were elevated in the antisense transfected cells. Most dramatic was the >10-fold upregulation of osteopontin and ALP in the antisense transfected cells. The dramatic effects of REMODELIN on osteopontin expression were further analyzed using a luciferase assay. NIH3T3 cells were cotransfected with a construct in which the osteopontin promoter driving luciferase activity (provided by Dr. Liaw) and the REMODELIN expression construct under the control of the CMV promoter. As shown in Figure 6, NIH3T3 cells express endogenous osteopontin. Compared to vector transfected cells, REMODELIN decreased luciferase activity by 80% (Figure 7). As discussed herein, REMODELIN affects bone formation, and thus,

it was of interest to determine the effect of REMODELIN on *Cbfa1*-dependent activity of the osteocalcin promoter. NIH3T3 and MC3T3-E1 cells were cotransfected with an osteocalcin-luciferase construct (containing OSE2 and others, provided by Dr. Karsenty), *Cbfa1*, and REMODELIN (or empty vector). REMODELIN completely inhibited the *Cbfa1*-mediated increase in osteocalcin-driven luciferase activity (Figure 7).

Bacterial expression of REMODELIN

REMODELIN protein with a 6x histidine tag was expressed in E. coli, and the recombinant protein was characterized by immunoblotting. Under non-reducing conditions, bands of 74, 49, and 25 kDa reacted with anti-his tag antibody (Figure 8, lane 1). Under reducing conditions, his-tagged recombinant REMODELIN protein runs as a single band of approximately 28 kDa (Figure 8, lanes 2 and 3) which is similar in size to the native protein detected on immunoblots (Figure 5A). Without wishing to be bound to any particular theory, these data suggest that REMODELIN is able to form dimers and trimers. These data indicate that REMODELIN is probably not glycosylated and it is therefore likely that the recombinant protein has similar properties as the native REMODELIN which make it suitable for in vitro studies (described herein). The 28 kDa band in lane 1 probably represents reduced REMODELIN (compare with Figure 8, lanes 2 and 3).

Effects of REMODELIN overexpression in *Xenopus laevis*

The biological effects of REMODELIN overexpression on *Xenopus laevis* development were studied using injection of REMODELIN mRNA into frog embryos. For injection experiments, a dose of 5 ng of either the long form of REMODELIN or the short form of REMODELIN mRNA was injected into embryos at the 2 cell stage. Controls embryos were injected with an equal volume of empty vehicle or lacZ mRNA.

Injection of both $REMODELIN_L$ and $REMODELIN_S$ disturbed normal embryonic development. In general, the percentage of oocytes exhibiting disturbed development was significantly higher in the oocytes injected with the short form (close

to 100%) while the long form of REMODELIN showed fewer malformed embryos. Without wishing to be bound by any particular theory, these data may indicate that the short form of REMODELIN is translated into protein *in vivo*.

In stage 17 embryos, there was a difference between lacZ-injected (Figure 9A, left 2 embryos) and REMODELIN-injected embryos (Figure 9A, right 2 embryos) indicating inhibition of blastopore closure.

At stage 34, control-injected embryos exhibited normal development (Figure 9B), however, REMODELIN-injected embryos displayed a number of defects (Figure 9C). The REMODELIN-injected embryos were smaller and were often distorted exhibiting abnormal development of the head. Due to failure of closure of the neural folds, fusion of the neurectoderm did not occur (Figure 9D). Other malformations included development of a split tail (Figure 9E). Several separate injection experiments were performed with similar results. This phenotype is remarkably similar to that of embryos injected with mRNA for dominant-negative FGF receptor constructs (Neilson and Friesel, 1996, J. Biol. Chem. 271:250497-25057).

The effect of REMODELIN on mesoderm induction was further assessed in that REMODELIN or control RNA was injected at the 2 cell stage and the embryos were allowed to develop to the blastula stage, at which time the animal pole ectoderm (animal caps) were dissected. Uninjected animal caps incubated in the presence of 200 ng/ml of FGF-1 elongated in a manner consistent with mesoderm induction while control-injected animal caps did not. Animal caps from embryos injected with REMODELIN and incubated in the presence of FGF-1 resembled animal caps incubated without FGF-1. This indicates that REMODELIN was able to block FGF-induced mesoderm formation.

Further, an important experiment assessed whether secretion of REMODELIN is necessary for function as follows. Using PCR, a construct comprising a deletion of the first 32 amino acids that have the potential for being either a cleavable signal peptide or a transmembrane domain, was designed (SEQ ID NO:9, Figure 10). The construct was cloned into the PCS2+ vector (American Type Culture Collection, Manassas, VA) and RNA was injected into frog embryos at the 2 cell stage. The resulting phenotype was similar to the one seen in REMODELIN$_S$-injected

embryos both in severity as well as frequency. Without wishing to be bound by any particular theory, these results suggest that it is likely that REMODELIN is not a secreted protein and that the putative N-myristoylation sites can anchor the protein in the cell membrane if required for biological function(s).

Function of REMODELIN in MC3T3 cells

MC3T3 cells were transfected with control vector (pcDNA3.1myc/his, Invitrogen, Carlsbad, CA) or with full length rat REMODELIN cDNA in an antisense orientation. Stably transfected clonal cell lines were then obtained and used in cell proliferation assays.

MC3T3-E1 cells were transfected with an antisense REMODELIN expression construct and stably transfected cell lines were established. Immunoblotting of clonal cell lines demonstrated that levels of REMODELIN protein in antisense transfectants were undetectable, as compared with normal levels of REMODELIN protein observed in vector transfectants (Figure 11).

Morphology of the cells was determined using phase contrast microscopy for both vector transfected (Figures 12A through 12C) and antisense REMODELIN transfected cells. As depicted in Figures 12D through 12I, antisense REMODELIN transfected cells exhibited a distinctly altered phenotype demonstrating less adhesion to the substratum and reduced cell-cell contacts. More specifically, antisense transfected cells were elongated and fibroblastic in appearance (Figures 12D-I). This is in contrast to control vector transfected cells, which exhibited a cobblestone morphology (Figures 12D through 12I).

The data disclosed herein also demonstrate that there were increased numbers of dead cells and cell debris in the antisense transfected cells indicating, without wishing to be bound by any particular theory, increased cell turnover in REMODELIN antisense transfected cells.

Increased cell turnover was determined by establishing growth curves of the clones (Figure 13A) and measuring [^{3}H]-thymidine incorporation in the clones (Figure 13B) in parallel experiments. Even though cell counts were similar between control and antisense REMODELIN transfected cells at all time points, [^{3}H]-thymidine

incorporation was significantly higher in the antisense REMODELIN transfected cells at all time points examined (Figure 13B). Increased cell turnover in the antisense transfected cells indicates that cell viability is reduced by shortening the cell life span. Together with the altered adhesion phenotype of the cells, the data suggests, without wishing to be bound by any particular theory, that REMODELIN is involved in cell-matrix and cell-cell interaction(s).

Expression of REMODELIN in transgenic mice

Transgenic mice were generated in which the coding region of REMODELIN was under the control of the CMV promoter. Breeding of a REMODELIN transgenic female with a REMODELIN transgenic male gave rise to mouse pups that exhibited hemorrhaging in the hip and shoulder regions. The bleeding appeared to originate from the long bones (Figures 14A and 15A), as fractures of the humerus (Figure 15B) and femur (Figure 15C) were evident. In one instance, bleeding also occurred in smaller bones of the foot.

X-ray examination of the skeleton revealed that all transgenic mice were smaller than corresponding non-transgenic mice (Figure 14B). This was particularly evident in the long bones.

Dissection of the dorsal skin revealed protrusion of the spinal cord similar to a phenotype seen in *spina bifida* disorders (Figure 14C).

Skeletal preparations of the control and transgenic pups were prepared using standard methods in order to further investigate the skeletal abnormalities. The skeletal preparations revealed a striking decrease in Alcian blue staining affecting all sites of cartilage in transgenic mice. The spinal column (Figures 15D and 15E) revealed a virtual absence of Alcian blue staining. Alcian blue, which binds to proteoglycans, identifies the areas of cartilage formation. The lack of Alcian blue thus indicates a marked reduction in the proteoglycan content of the cartilage. Alcian blue staining was also reduced in the extremities, particularly the distal phalanges. The severity of the phenotype was variable, as in some cases, the offspring from a REMODELIN transgenic/wildtype cross gave rise to severely affected mutants that died perinatally (Figures 15H through 15L). These mice were dwarfs with severe

skeletal abnormalities affecting all bones (Figures 15H and 15I). In addition, separation of the skin occurred which is reminiscent of dystrophic epidermolysis bullosea (Figures 15H and 15L).. X-ray examination of these transgenics showed extremely reduced bone density (lack of mineralization) and deformed bones (Figure 15I). Masson's trichrome stained sections exhibited dramatically reduced collagen matrix (blue) in many organs including skull bone (Figure 15J) and ribs (Figure 15K). separation of the epidermis from the dermis also appeared to result from a deficient collagen matrix (Figure 15L). Preliminary analysis indicated that chondrocyte maturation appeared normal.

Mineralized bone of skeletal preparations was stained using Alizarin Red (pink color depicted as darker gray) and cartilage was stained using Alcian Blue (blue color depicted as lighter gray) (Figures 16A through 16J). The data disclosed herein demonstrate that there was a striking decrease in cartilage formation affecting all sites of cartilage generation including the extremities, particularly the distal phalanges (Figure 16A compared with Figure 16B). Cartilage was completely missing from the vertebra and intervertebral joints in the REMODELIN transgenic mice (Figure 16D) compared with normal pups (Figure 16C), leaving the posterior parts of the vertebrae and the intervertebral joints without cartilage.

The absence of cartilage in the posterior parts of the vertebra surrounding the spinal cord is the most likely reason for the protrusion of the spinal cord leading the *spina bifida* phenotype. The anterior parts of the ribs exhibited strikingly reduced cartilage formation, which was most pronounced in the more caudal ribs (Figure 16F compared with Figure 16E).

Another finding was the marked decrease in the density of the mineralized bone which gave the flat bones of the skull a more transparent appearance (*compare* Figure 16H *with* Figure 16G). Without wishing to be bound by any particular theory, the decreased bone density is expected to result in weaker bones with increased tendency to fracture. Indeed, the hemorrhaging observed in the shoulder and hip regions was found to be the result of fractured long bones such as the humerus (Figures 16I and 16J) and femur.

For a better understanding of the phenotypes observed in REMODELIN transgenic mice and to clarify whether the abnormalities were the result of ectopic expression or overexpression in cell types that have endogenous levels of REMODELIN, the sites of transgene expression were characterized and compared with the expression pattern of endogenous REMODELIN protein. Polyclonal antibodies were generated in rabbits with the recombinant protein as antigen. One of the rabbits produced an antibody that recognized recombinant REMODELIN and native REMODELIN in lung tissue lysates on immunoblots with similar sensitivity and specificity as the peptide antibody described herein. In addition, the antibody was suitable for immunohistochemistry.

A protein A-purified IgG fraction was prepared from the antiserum and the corresponding preimmune serum was used to immunostain bone, skin and muscle sections from one day old normal and REMODELIN transgenic pups. In parallel, immunostaining on sections with the transgene specific anti-myc antibody was performed (Figures 17 and 18). Chondrocytes of the humerus head express endogenous REMODELIN mRNA (Figure 17A) and endogenous REMODELIN protein was expressed abundantly by chondrocytes (Figures 17B and 17C). However, REMODELIN protein expression decreased sharply in the hypertrophic cartilage zone as it reaches the osteogenic front so that the bone matrix is essentially devoid of endogenous REMODELIN protein (Figures 17B and 17C). Periosteal cells and osteoblasts expressed both REMODELIN mRNA (Figure 17D) and protein (Figure 17F) although at a lower level than chondrocytes. Expression of endogenous REMODELIN in osteocytes was not detectable (Figure 17F). The REMODELIN -myc transgene protein, however, was not expressed by chondrocytes (Figure 17G, upper right corner) but high levels were synthesized by osteocytes in the bone matrix (Figure 17H, arrowheads). Lower levels of REMODELIN -myc protein were also found in osteoblasts. Controls for immunostaining were performed with preimmune IgG, and these revealed very little nonspecific background (Figure 17I). Thus, in the transition from the cartilage matrix to the bone matrix, endogenous REMODELIN and the REMODELIN -myc transgene had inverse expression patterns (compare Figures 17B and 17G). Without wishing to be bound to any particular theory, the endogenous

REMODELIN expression pattern supports the idea that REMODELIN functions as an inhibitor of mineralization, and the expression pattern of the transgene provides an explanation for the normal development of the cartilage in the transgenic mice. With regard to osteoblasts, the transgenic mice provide an in vivo overexpression model.

Immunohistochemistry performed on skin sections from normal one day old mouse pups showed no REMODELIN protein detectable by the anti-REMODELIN IgG (Figure 18A). It should be emphasized, however, that skin wound repair is associated with high levels of REMODELIN mRNA expression by dermal fibroblasts. High levels of the REMODELIN -myc transgene were expressed by keratinocytes in the epidermis as determined by immunohistochemistry with the anti-myc antibody (Figure 18B). The dermis of these transgenic mice was characterized by an unusually loose connective tissue (Figure 18C) and hair follicles were often absent or poorly developed. The abnormalities in the skin could explain the DEB phenotype. Skeletal muscle in normal mice revealed REMODELIN immunoreactivity in the muscle fibers (Figure 18D) and high levels of the transgene were also detected in skeletal muscle with the anti-myc antibody (Figure 18G and Fig. 17G). Interestingly, the muscle fiber bundles in the transgenics were often shorter and less densely packed (Figures 18H and 18I). In addition, the fiber bundles often formed circular structures that appeared hollow in the center (Figures 18G, 18H, and 18I). These findings indicate the presence of a severe myopathy in REMODELIN transgenic mice. With regard to skeletal muscle, the transgenic mice provide an in vivo overexpression model. Expression of the transgene in skeletal muscle is consistent with data presented herein that demonstrates activity of the CMV promoter in this tissue.

The data disclosed herein using transgenic mice indicate that REMODELIN plays an important role in bone growth. Similar to the frog embryo injection experiments wherein REMODELIN mRNA mediated a failure of dorsal closure, the mouse transgenics also exhibit *spina bifida*-like defects of the spinal column.

These findings demonstrate that altered expression of REMODELIN is affecting vital mechanisms of bone formation. In particular, increased REMODELIN expression inhibits cartilage and bone formation resulting in reduced bone growth and

bone mineralization which gives rise to more fragile bones. Furthermore, without wishing to be bound by any particular theory, the data disclosed herein suggest that inhibition of REMODELIN expression can lead to the opposite phenotype with increased cartilage formation and increased bone density and strength. The level of REMODELIN expression could thus be a predictor of bone formation, bone density and bone strength. Further, inhibiting REMODELIN expression may be useful for diseases, disorders, or conditions associated with decreased bone density, bone formation and bone strength such as, but not limited, osteoporosis, and the like.

The data disclosed herein demonstrate that REMODELIN is expressed selectively in settings where remodeling occurs, *i.e.*, skin incisional wounds, bone, and the like. Without wishing to be bound by any particular theory, these data suggest that the role of REMODELIN is not restricted to the vasculature but instead REMODELIN expression is be relevant to events in wound healing in general, including bone formation, bone density and bone strength.

Wound healing is characterized by the formation of granulation tissue from connective tissue surrounding the damaged area and its components are inflammatory cells, fibroblasts and myofibroblasts (smooth muscle α-actin positive). As the wound closes and evolves into a scar, there is an important decrease in cellularity and a specific disappearance of myofibroblasts (Ducy et al., 2000, Science 289:1501-1504; Giachelli et al., 1993, J. Clin. Invest. 92:1686-1696). This cell loss has been shown to occur by apoptosis (Giachelli et al., 1993, J. Clin. Invest. 92:1686-1696). Failure to decrease this cellularity may contribute to hypertrophic scarring and keloid formation (Balica et al., 1997, Circulation 95:1954-1960; Bostrom et al., 1993, J. Clin. Invest. 91:1800-1809). The response of the adventitia to balloon injury (Bostrom et al., 1995, Amer. J. Cardiol. 75:88B-91B), and the response of the myocardium to infarction are very similar (Luo et al., 1997, Nature 386:78-81) with early accumulation of myofibroblasts and subsequent loss of cells by apoptosis as disclosed elsewhere herein resulting in an acellular matrix-rich structure. It should be emphasized that, as demonstrated by data disclosed elsewhere herein, REMODELIN is induced in these myofibroblasts while it is not expressed in the dedifferentiated smooth muscle cells (SMC) of the neointima. While smooth muscle α-actin is down-regulated

in the dedifferentiated, proliferating SMC of the neointima, it is induced in the myofibroblasts of the adventitia (Bostrom et al., 1995, Amer. J. Cardiol. 75:88B-91B), but is lost from the adventitial cells within 2 weeks after injury.

REMODELIN expression may play a role in other clinically relevant situations of fibrosis, including liver fibrosis and pulmonary fibrosis. In liver fibrosis, apoptosis of hepatic stellate cells has been implicated in the fibrotic process and targeting apoptosis may be a promising strategy for antifibrotic therapies (Cales et al., 1998, Biomed. Pharmacother. 52:259-263). TGF-ß has been identified as the major factor responsible for fibrosis in the bleomycin-induced lung fibrosis model Wang et al., 1999, Thorax 54:805-812). Finally, since REMODELIN is expressed in developing bone it should be mentioned that apoptosis is an integral part of endochondral ossification and bone fracture healing with chondrocytes and osteoblasts undergoing apoptosis (Einhorn et al., 1998, Clin. Orthop. S7-21; Olmedo et al., 1999, J. Orthop. Trauma 13:356-362). The prominent expression of REMODELIN in developing bone further suggests, without wishing to be bound by any particular theory, that REMODELIN is involved in regulating calcification since inhibition of calcification is an important event in cell death (Kim, 1995, Scanning Microsc. 9:1137-1178; Kockx et al, 1998, Arterioscler. Thromb. Vasc. Biol. 18:1519-1522).

As discussed herein, mutations in collagens are responsible for OI, Bethlem myopathy, and DEB (reviewed in Spranger et al., 1994, Eur. J. Pediatr., 153:56-65). Therefore, a reasonable hypothesis is that REMODELIN functions as a modulator of collagen matrices. Data presented herein make it clear that it is absolutely essential that the REMODELIN expression levels be tightly regulated for normal development. The importance of this molecule is further demonstrated in that it is the first non-collagen gene to produce an OI phenotype. Based on data presented herein, it is likely that REMODELIN is an endogenous modulator of collagen matrices. In that capacity, the molecule could function as an endogenous regulator of (anti)fibrotic responses. The inhibition of bone mineralization could be a consequence of a deficient collagen bone matrix that does not support mineralization. In addition, the potential role of REMODELIN as a signaling molecule needs to be addressed as it was able to inhibit osteopontin promoter activity and *Cbfa1*-dependent activity of the

osteocalcin promoter. Furthermore, reduced expression of collagen was seen in cells overexpressing REMODELIN (Figure 6). If REMODELIN would function solely by incorporation as a component of a collagenous matrix, it should be present in matrices of healed wounds. A completed repair process in response to angioplasty injury of an artery, however, is associated with low REMODELIN levels (Figure 5C, 28 day time point) similar to normal vessels.

In summary, the hypothesis of REMODELIN as a modulator of collagen matrices is based on the following findings: 1) increased REMODELIN expression in vitro is associated with decreased TGF-ß expression as well as reduced TGF-ß dependent gene expression (collagens I and III) in vivo and in vitro, 2) increased REMODELIN expression in vivo results in phenotypes reminiscent of collagen mutations found in OI, DEB, and myopathies, 3) in the absence of REMODELIN, expression of bone differentiation markers such as osteopontin and ALP are dramatically increased, and 4) the presence of a 36 aa domain with homology to the triple helical repeat region of collagens. The proposed hypothesis for the function of REMODELIN is based on insight derived mainly from in vivo overexpression as well as ectopic expression.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While the invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. An isolated nucleic acid encoding a mammalian REMODELIN, or a fragment thereof.

2. The isolated nucleic acid of claim 1, wherein said nucleic acid shares at least about 33% sequence identity with a nucleic acid encoding at least one of rat REMODELIN (SEQ ID NO:1), and a human REMODELIN (SEQ ID NO:3).

3. An isolated nucleic acid encoding a mammalian REMODELIN, wherein the amino acid sequence of said REMODELIN shares at least about 6% sequence identity with an amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

4. An isolated polypeptide comprising a mammalian REMODELIN.

5. The isolated polypeptide of claim 4, wherein said mammalian REMODELIN molecule shares at least about 6% sequence identity with an amino acid sequence of at least one of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:5.

6. The nucleic acid of claim 1, said nucleic acid further comprising a nucleic acid encoding a tag polypeptide covalently linked thereto.

7. The nucleic acid of claim 6, wherein said tag polypeptide is selected from the group consisting of a green fluorescent protein tag polypeptide, an influenza virus hemagglutinin tag polypeptide, a myc tag polypeptide, a glutathione-S-transferase tag polypeptide, a myc-pyruvate kinase tag polypeptide, a His6 tag polypeptide, a FLAG tag polypeptide, and a maltose binding protein tag polypeptide.

8. The nucleic acid of claim 1, said nucleic acid further comprising a nucleic acid specifying a promoter/regulatory sequence operably linked thereto.

9. A vector comprising the nucleic acid of claim 1.

10. The vector of claim 9, said vector further comprising a nucleic acid specifying a promoter/regulatory sequence operably linked thereto.

11. A recombinant cell comprising the isolated nucleic acid of claim 1.

12. A recombinant cell comprising the vector of claim 9.

13. An isolated nucleic acid complementary to the nucleic acid of claim 1, said complementary nucleic acid being in an antisense orientation.

14. The isolated nucleic acid of claim 13, wherein said nucleic acid shares at least about 33% identity with a nucleic acid complementary with a nucleic acid having the sequence of at least one of a rat REMODELIN molecule (SEQ ID NO:1), and a human REMODELIN molecule (SEQ ID NO:3).

15. A recombinant cell comprising the isolated nucleic acid of claim 13.

16. An antibody that specifically binds with a mammalian REMODELIN molecule polypeptide, or a fragment thereof.

17. The antibody of claim 16, wherein said antibody is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, a humanized antibody, a chimeric antibody, and a synthetic antibody.

18. A composition comprising the antibody of claim 16 and a pharmaceutically-acceptable carrier.

19. A composition comprising the isolated nucleic acid of claim 13 and a pharmaceutically-acceptable carrier.

20. A composition comprising the isolated nucleic acid of claim 1 and a pharmaceutically-acceptable carrier.

21. A composition comprising the isolated polypeptide of claim 4 and a pharmaceutically-acceptable carrier.

22. A transgenic non-human mammal comprising the isolated nucleic acid of claim 1.

23. A method of treating a disease mediated by abnormal expression of a REMODELIN molecule in a human, said method comprising administering to a human patient afflicted with a disease mediated by abnormal expression of a REMODELIN molecule a REMODELIN molecule expression-inhibiting amount of the composition of claim 19.

24. The method of claim 23, wherein said disease is selected from the group consisting of impaired wound healing, fibrosis of an organ, ectopic ossification, and hypertrophic scar formation.

25. A method of diagnosing arterial restenosis in a mammal, said method comprising obtaining a biological sample from said mammal, assessing the level of REMODELIN in said biological sample, and comparing the level of REMODELIN in said biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with arterial restenosis, wherein a higher level of REMODELIN in said biological sample from said mammal compared with the level of REMODELIN in said biological sample from said like mammal is an

indication that said mammal is afflicted with arterial restenosis, thereby diagnosing arterial restenosis in said mammal.

26. The method of claim 25, wherein said biological sample is selected from the group consisting of a blood vessel sample, and a damaged tissue sample.

27. A method of diagnosing negative remodeling in a mammal, said method comprising obtaining a biological sample from said mammal, assessing the level of REMODELIN in said biological sample, and comparing the level of REMODELIN in said biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with negative remodeling, wherein a higher level of REMODELIN in said biological sample from said mammal compared with the level of REMODELIN in said biological sample from said like mammal is an indication that said mammal is afflicted with negative remodeling, thereby diagnosing negative remodeling in said mammal.

28. A method of diagnosing fibrosis in a mammal, said method comprising obtaining a biological sample from said mammal, assessing the level of REMODELIN in said biological sample, and comparing the level of REMODELIN in said biological sample with the level of REMODELIN in a biological sample obtained from a like mammal not afflicted with fibrosis, wherein a higher level of REMODELIN in said biological sample from said mammal compared with the level of REMODELIN in said biological sample from said like mammal is an indication that said mammal is afflicted with fibrosis, thereby diagnosing fibrosis in said mammal.

29. A method of identifying a compound that affects expression of REMODELIN in a cell, said method comprising contacting a cell with a test compound and comparing the level of REMODELIN expression in said cell with the level of REMODELIN expression in an otherwise identical cell not contacted with said test compound, wherein a higher or lower level of REMODELIN expression in said cell contacted with said test compound compared with the level of REMODELIN

expression in said otherwise identical cell not contacted with said test compound is an indication that said test compound affects expression of REMODELIN in a cell.

30. A compound identified by the method of claim 29.

31. A method of identifying a compound that reduces expression of REMODELIN in a cell, said method comprising contacting a cell with a test compound and comparing the level of REMODELIN expression in said cell with the level of REMODELIN expression in an otherwise identical cell not contacted with said test compound, wherein a lower level of REMODELIN expression in said cell contacted with said test compound compared with the level of REMODELIN expression in said otherwise identical cell not contacted with said test compound is an indication that said test compound reduces expression of REMODELIN in a cell.

32. A compound identified by the method of claim 31.

33. A method of identifying a compound that affects TGF-β signaling, said method comprising contacting a cell with a test compound and comparing the level of REMODELIN expression in said cell with the level of REMODELIN expression in an otherwise identical cell not contacted with said test compound, wherein a higher or lower level of REMODELIN expression in said cell contacted with said test compound compared with the level of REMODELIN expression in said otherwise identical cell not contacted with said test compound is an indication that said test compound affects TGF-β signaling in a cell.

34. A kit for alleviating a disease mediated by abnormal expression of a REMODELIN in a human, said kit comprising a REMODELIN expression-inhibiting amount of the composition of claim 19, said kit further comprising an applicator, and an instructional material for the use thereof.

35. The kit of claim 34, wherein said disease is selected from the group consisting of negative remodeling, arterial restenosis, vessel injury, fibrosis.

36. A kit for alleviating a disease mediated by abnormal expression of a REMODELIN in a human, said kit comprising a REMODELIN expression-inhibiting amount of the composition of claim 20, said kit further comprising an applicator, and an instructional material for the use thereof.

37. A kit for treating a bone disease in a mammal, said kit comprising a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, said kit further comprising an applicator, and an instructional material for the use thereof.

38. A kit for treating a cartilage disease in a mammal, said kit comprising a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, said kit further comprising an applicator, and an instructional material for the use thereof.

39. A kit for inhibiting tissue calcification, said kit comprising a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, said kit further comprising an applicator, and an instructional material for the use thereof.

40. The kit of claim 39, wherein said tissue calcification is calcification of a transplant.

41. The kit of claim 40, wherein said transplant is a heart valve transplant.

42. A method of increasing REMODELIN expression in a mammal, said method comprising administering a REMODELIN expression increasing amount

of TGF-β to said mammal, thereby increasing REMODELIN expression in said mammal.

43. A method of reducing REMODELIN expression in a mammal, said method comprising administering a REMODELIN expression reducing amount of TGF-β receptor type II to said mammal, thereby inhibiting signaling via TGF-β receptor type II and reducing expression of REMODELIN in said mammal.

44. A method of affecting cellular gene expression in a mammal, said method comprising administering a nucleic acid encoding REMODELIN to said mammal, thereby affecting cellular gene expression in said mammal.

45. The method of claim 44, wherein said cellular gene is selected from the group consisting of TGF-β1, collagen IIIα1, osteopontin, biglycan, alkaline phosphatase, and bone morphogenic protein 4.

46. The method of claim 45, wherein said expression of osteopontin is dependent on Cbfa1.

47. A method of affecting cellular gene expression in a mammal, said method comprising administering a nucleic acid antisense to a nucleic acid encoding REMODELIN to said mammal, thereby affecting cellular gene expression in said mammal.

48. A method of treating bone disease in a mammal in need of such treatment, said method comprising administering to a mammal afflicted with said bone disease a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, thereby inhibiting REMODELIN expression and treating said bone disease in said mammal.

49. The method of claim 48, wherein said bone disease is osteogenesis imperfecta.

50. A method of treating cartilage disease in a mammal in need of such treatment, said method comprising administering to a mammal afflicted with said cartilage disease a REMODELIN expression-inhibiting amount of an inhibitor of REMODELIN expression, thereby inhibiting REMODELIN expression and treating said cartilage disease in said mammal.

51. The method of claim 50, wherein said collagen disease is selected from the group consisting of osteogenesis imperfecta (OI), dystrophic epidermolysis bullosea (DEB), and Bethlem myopathy.

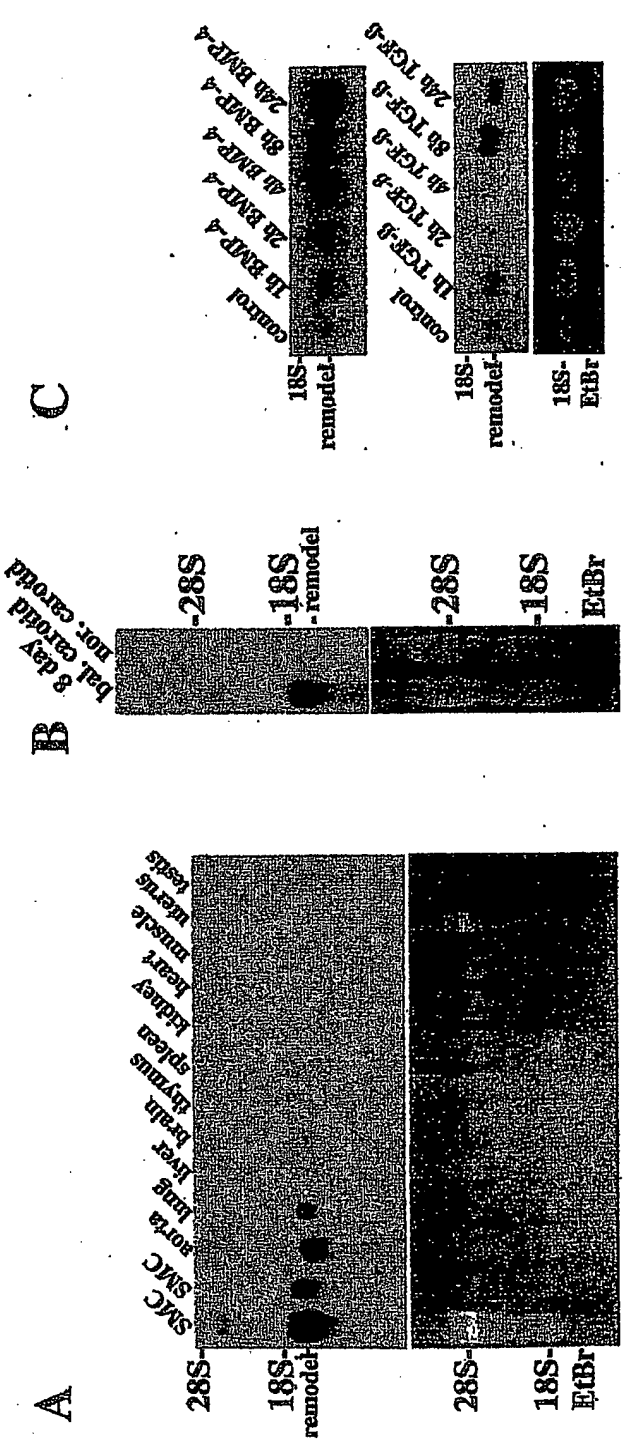
52. A method of diagnosing a bone disease in a mammal, said method comprising obtaining a biological sample from said mammal, assessing the level of REMODELIN in said biological sample, and comparing the level of REMODELIN in said biological sample with the level of REMODELIN in a biological sample obtained from an otherwise identical mammal not afflicted with bone disease, wherein a higher level of REMODELIN in said biological sample from said mammal compared with said level of REMODELIN in said biological sample from said like mammal is an indication that said mammal is afflicted with bone disease, thereby diagnosing said bone disease in said mammal.

53. The method of claim 52, wherein said bone disease is osteogenesis imperfecta.

54. A method of diagnosing a collagen disease in a mammal, said method comprising obtaining a biological sample from said mammal, assessing the level of REMODELIN in said biological sample, and comparing the level of REMODELIN in said biological sample with the level of REMODELIN in a biological sample obtained from an otherwise identical mammal not afflicted with a collagen

disease, wherein a higher level of REMODELIN in said biological sample from said mammal compared with said level of REMODELIN in said biological sample from said like mammal is an indication that said mammal is afflicted with a collagen disease, thereby diagnosing said collagen disease in said mammal.

55. The method of claim 54, wherein said collagen disease is selected from the group consisting of osteogenesis imperfecta (OI), dystrophic epidermolysis bullosea (DEB), and Bethlem myopathy.


A
28S-
18S-remodel
SMC
SMC
aorta
lung
liver
br-lab
thymus
spleen
kidney
heart
muscle
uterus
testis
28S-
18S-EtBr
B
8 day
bal. carotid
nor. carotid
28S
18S-remodel
28S
18S
EtBr
C
18S-remodel
control
1h BMP-4
2h BMP-4
4h BMP-4
8h BMP-4
24h BMP-4
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control
1h TGF-β
2h TGF-β
4h TGF-β
8h TGF-β
24h TGF-β
18S-EtBr

FIG. 1

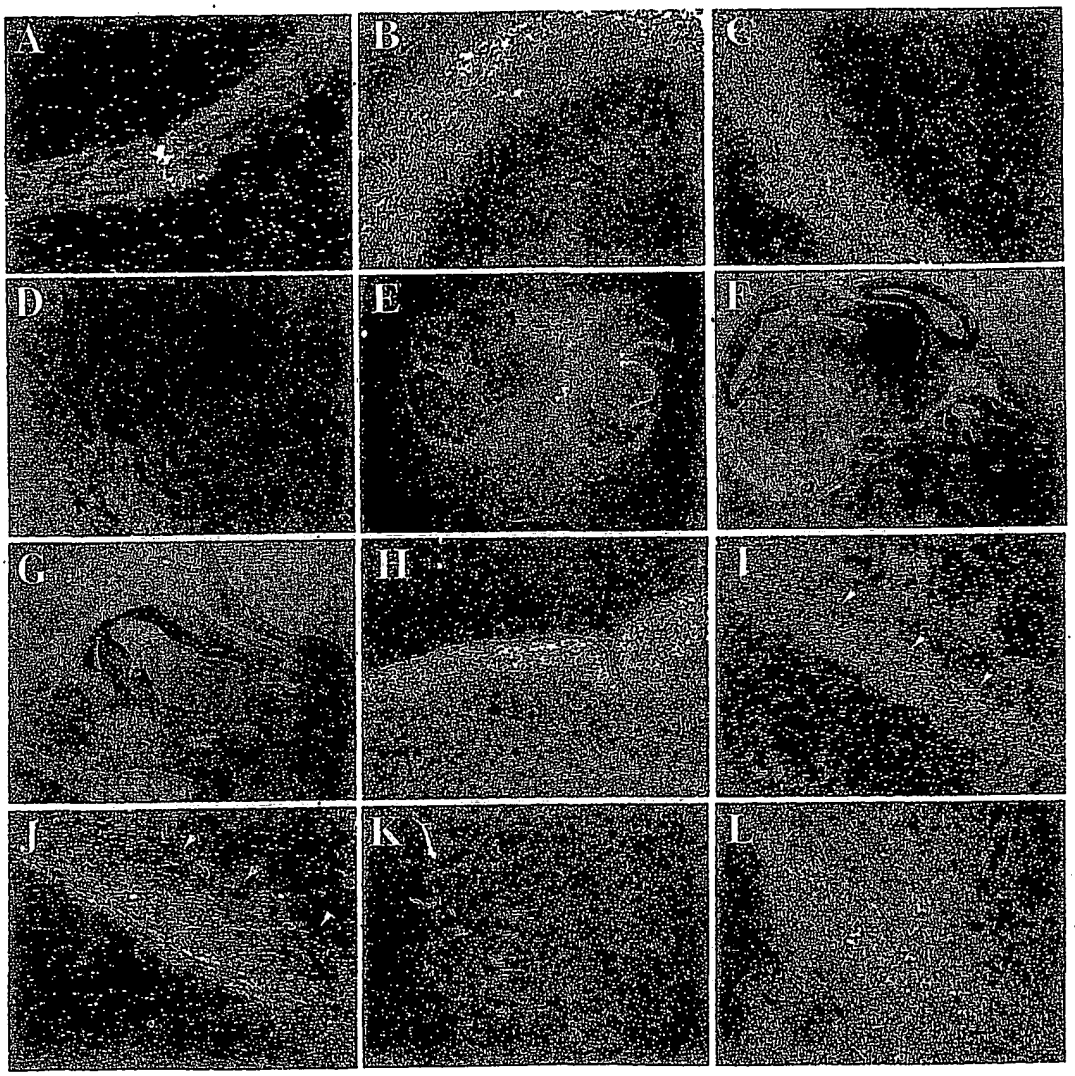


FIG. 2

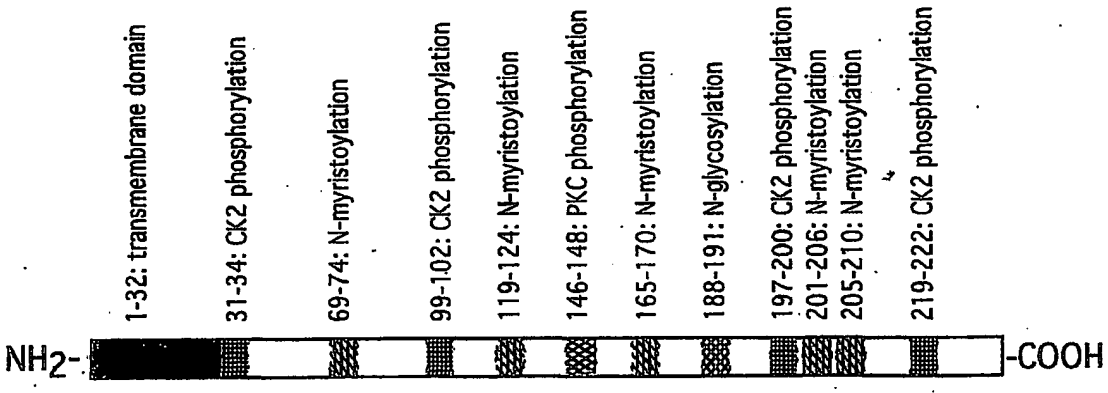

1-32: transmembrane domain
31-34: CK2 phosphorylation
69-74: N-myristoylation
99-102: CK2 phosphorylation
119-124: N-myristoylation
146-148: PKC phosphorylation
165-170: N-myristoylation
188-191: N-glycosylation
197-200: CK2 phosphorylation
201-206: N-myristoylation
205-210: N-myristoylation
219-222: CK2 phosphorylation
NH2-
-COOH

FIG. 3

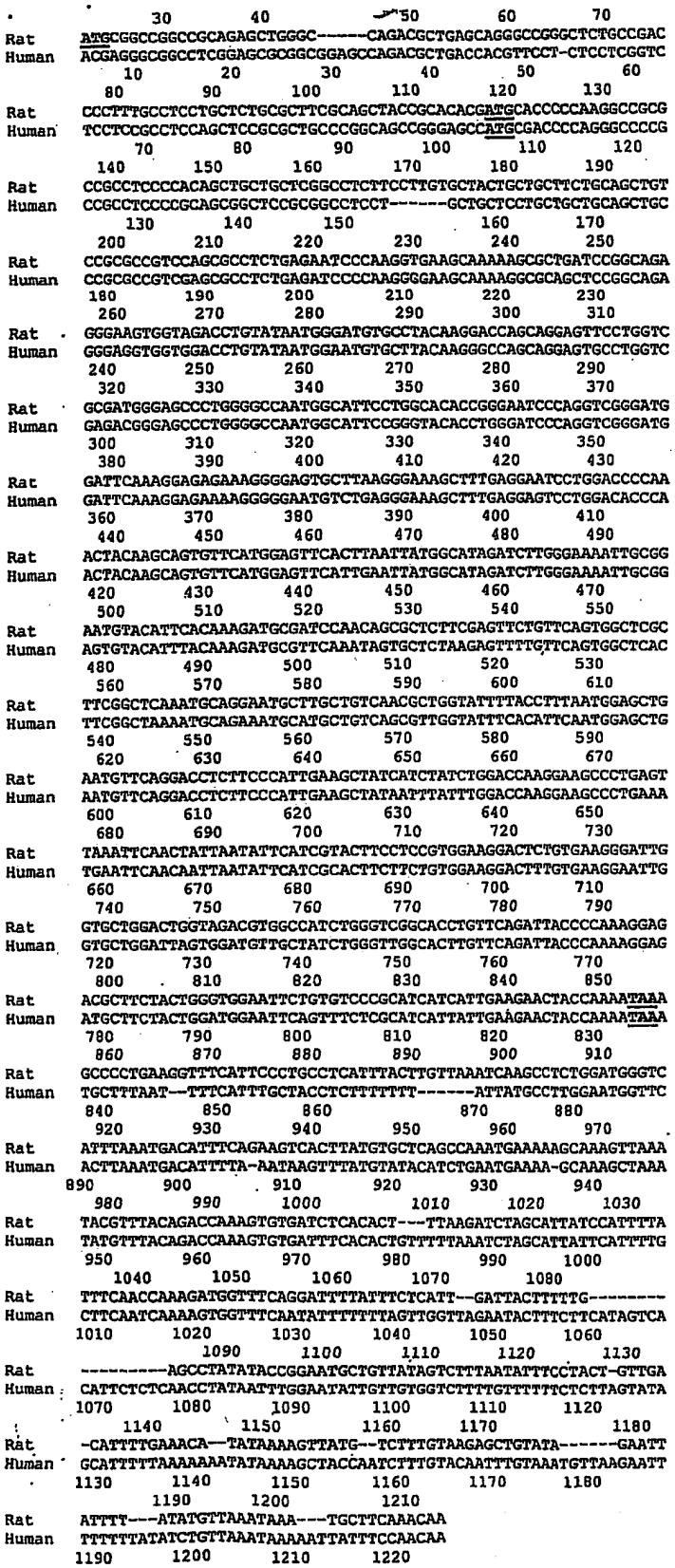


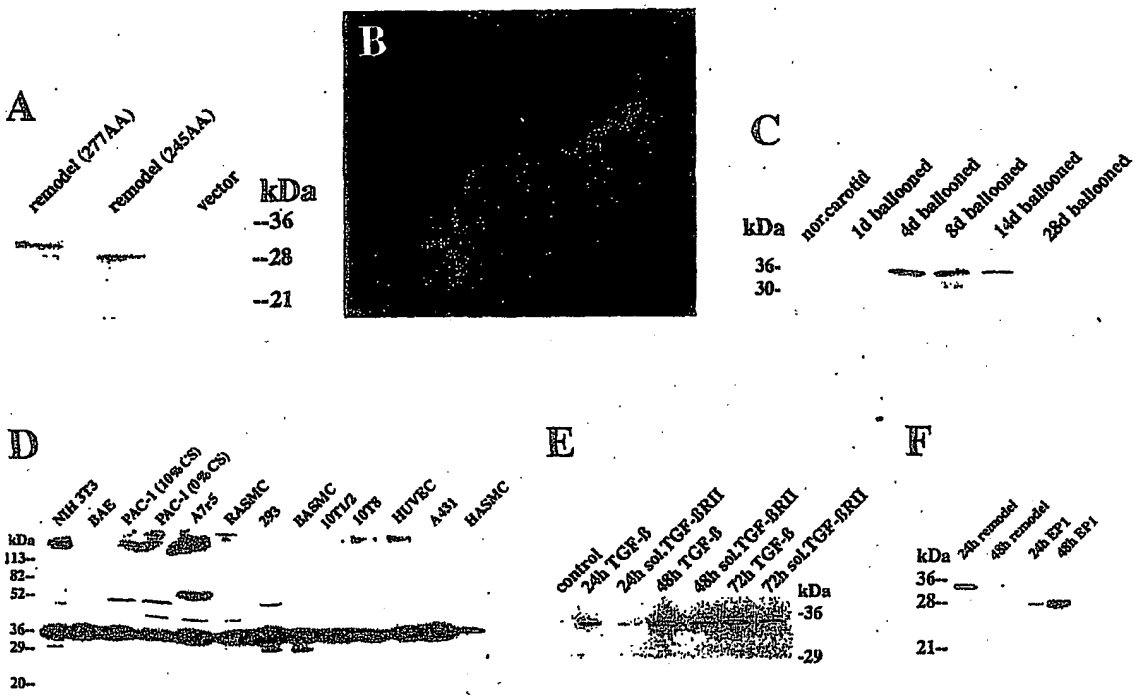
Figure 4A

```
Rat:    1    MHPQGRAASPQLLLGLFLVLLLLLQLSAPSSASENPKVKQKALIRQREVVDLYNGMCLQG   60
             M+PQG+AASPQ+L+GL+++LLLLLQL+APSSASE+PK+KQKA++RQREVVDLYNGMCLQG
Human:  1    MRPQGPAASPQRLRGL--LLLLLLQLPAPSSASEIPKGKQKAQLRQREVVDLYNGMCLQG   58

Rat:    61   PAGVPGRDGSPGANGIPGTPGIPGRDGFKGEKGECLRESFEESWTPNYKQCSWSSLNYGI   120
             PAGVPGRDGSPGANGIPGTPGIPGRDGFKGEKGECLRESFEESWTPNYKQCSWSSLNYGI
Human:  59   PAGVPGRDGSPGANGIPGTPGIPGRDGFKGEKGECLRESFEESWTPNYKQCSWSSLNYGI   118

Rat:    121  DLGKIAECTFTKMRSNSALRVLFSGSLRLKCRNACCQRWYFTFNGAECSGPLPIEAIIYL   180
             DLGKIAECTFTKMRSNSALRVLFSGSLRLKCRNACCQRWYFTFNGAECSGPLPIEAIIYL
Human:  119  DLGKIAECTFTKMRSNSALRVLFSGSLRLKCRNACCQRWYFTFNGAECSGPLPIEAIIYL   178

Rat:    181  DQGSPELNSTINIHRTSSVEGLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIII   240
             DQGSPE+NSTINIHRTSSVEGLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIII
Human:  179  DQGSPEMNSTINIHRTSSVEGLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIII   238

Rat:    241  EELPK  245
             EELPK
Human:  239  EELPK  243
```

Figure 4B

MRPAAELGQTLSRAGLCRPLCLLLCASQLPHTMHPQGRAASPQLLLGLFLVLLLLLQL
SAPSSASENPKVKQKALIRQREVVDLYNGMCLQGPAGVPGRDGSPGANGIPGTPGIPG
RDGFKGEKGECLRESFEESWTPNYKQCSWSSLNYGIDLGKIAECTFTKMRSNSALRVL
FSGSLRLKCRNACCQRWYFTFNGAECSGPLPIEAIIYLDQGSPELNSTINIHRTSSVE
GLCEGIGAGLVDVAIWVGTCSDYPKGDASTGWNSVSRIIIEELPK

FIG. 4C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remodel (277AA)
remodel (245AA)
vector
kDa
-36
-28
-21
B
C
kDa
nor.carotid
1d ballooned
4d ballooned
8d ballooned
14d ballooned
28d ballooned
36-
30-
D
NIH 3T3
BAE
PAC-1 (10% CS)
PAC-1 (0% CS)
A7r5
RASMC
293
BASMC
10T1/2
19T8
HUVEC
A431
HASMC
kDa
113--
82--
52--
36--
29--
20--
E
control
24h TGF-β
24h sol.TGF-βRII
48h TGF-β
48h sol.TGF-βRII
72h TGF-β
72h sol.TGF-βRII
kDa
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-29
F
kDa
24h remodel
48h remodel
24h EP1
48h EP1
36--
28--
21--

Figure 5

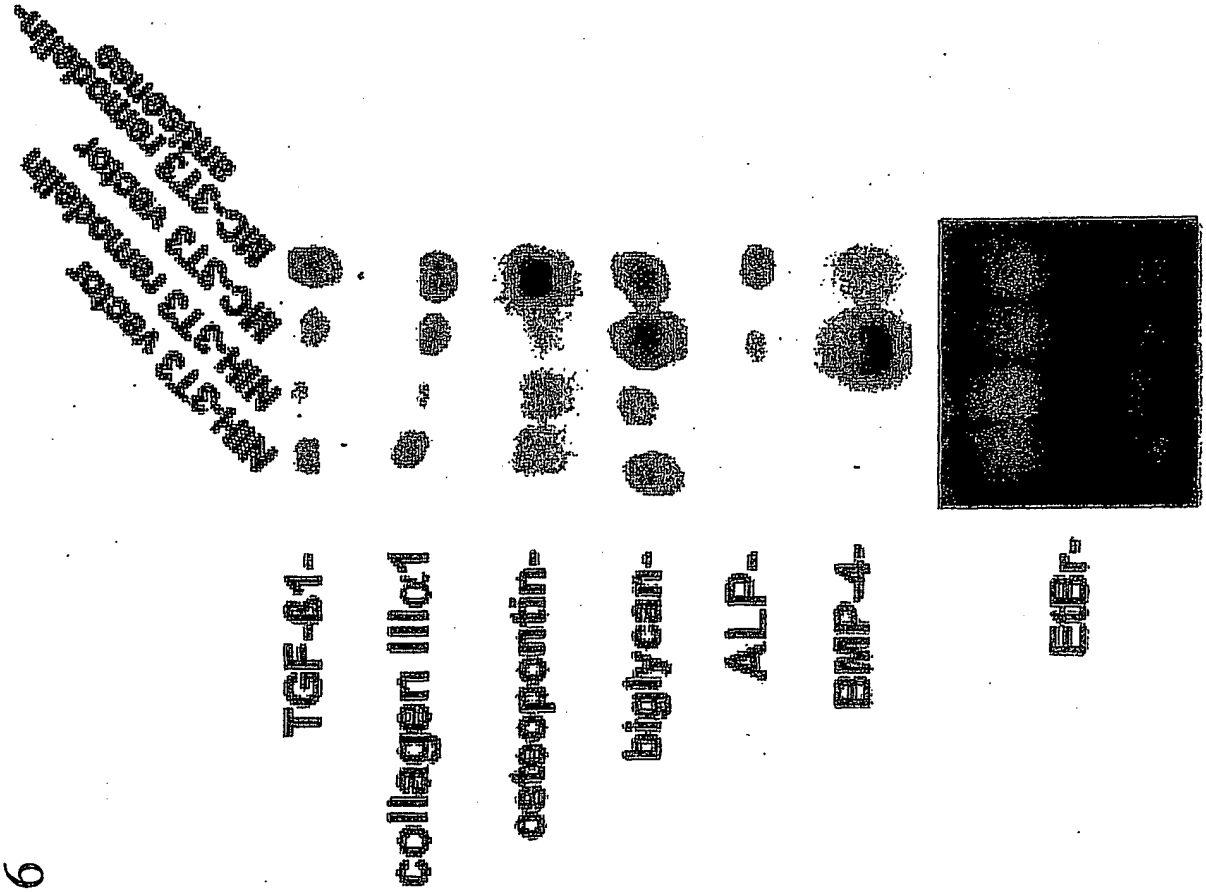
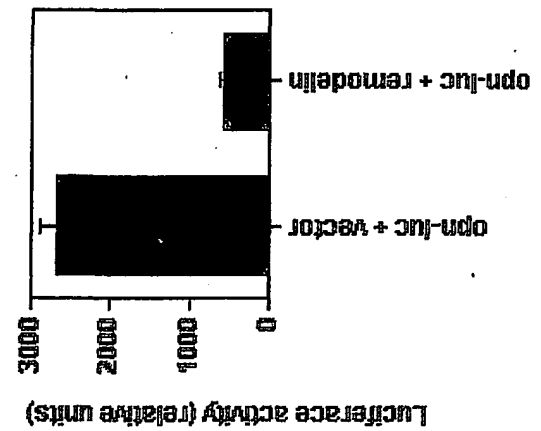

MMP-13 vector control
MMP-13 vector
MC-3T3 control
MC-3T3 vector
MMP-13 antisense
TGF-β1
collagen III α1
osteopontin
biglycan
ALP
BMP-4
EtBr

Figure 6


opn-luc + remodelin
opn-luc + vector
3000
2000
1000
0
Luciferace activity (relative units)

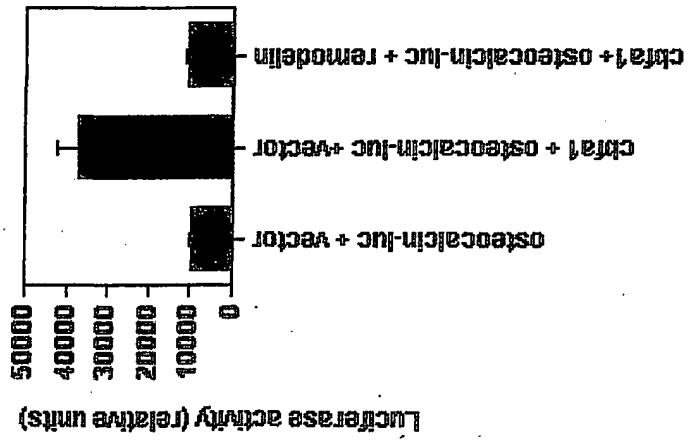

ctfra1+ osteocalcin-luc + remodelin
ctfra1 + osteocalcin-luc +vector
osteocalcin-luc + vector
50000
40000
30000
20000
10000
0
Luciferase activity (relative units)

Figure 7

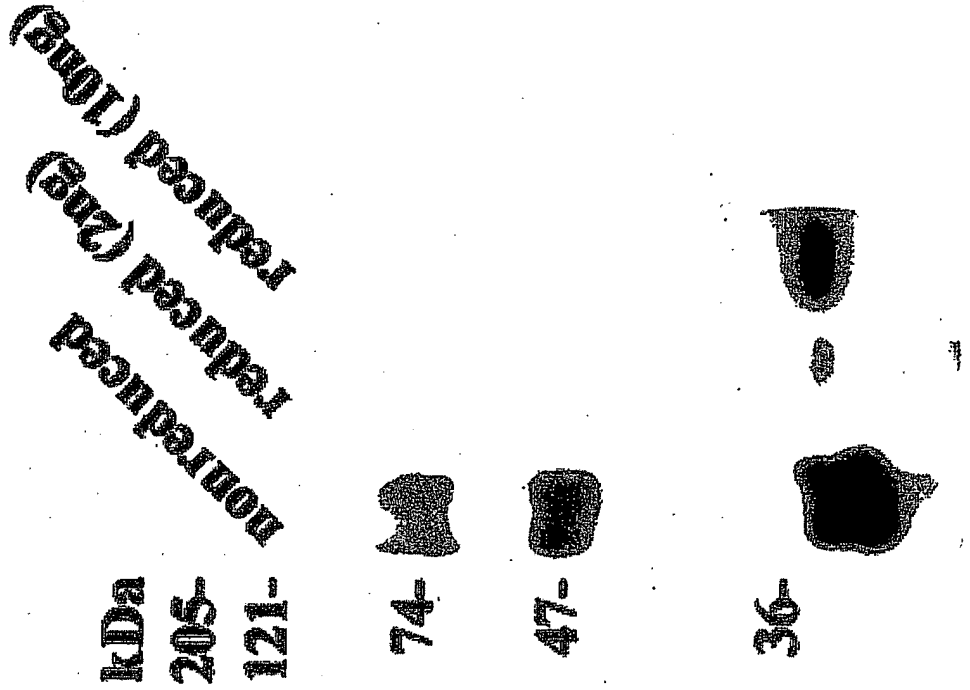


Figure 8

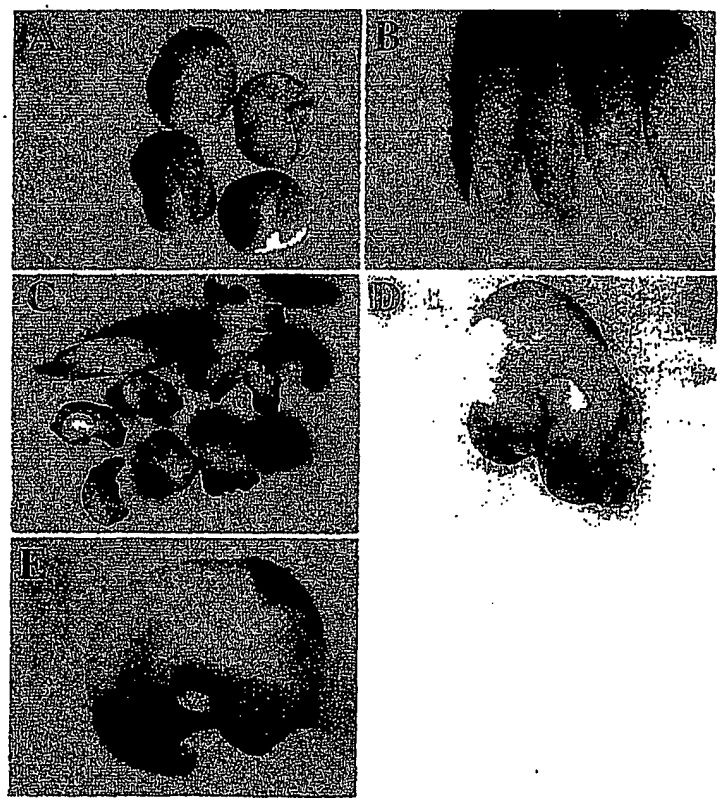


Figure 9

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ATG GCCCCCAAGG CCGCGCCGCC TCCCCACAGC TGCTGCTCGG CCTCTTCGTT GTGCTACTGC
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    CGCTGATCCG GCAGAGGGAA GTGGTAGACC TGTATAATGG GATGTGCCTA CAAGGACCAG
    CAGGAGTTCC TGGTCGCGAT GGGAGCCCTG GGGCCAATGG CATTCCTGGC ACACCGGGAA
    TCCCAGGTCG GGATGGATTC AAAGGAGAGA AAGGGGAGTG CTTAAGGGAA AGCTTTGAGG
    AATCCTGGAC CCCAAACTAC AAGCAGTGTT CATGGAGTTC ACTTAATTAT GGCATAGATC
    TTGGGAAAAT TGCGGAATGT ACATTCACAA AGATGCGATC CAACAGCGCT CTTCGAGTTC
    TGTTCAGTGG CTCGCTTCGG CTCAAATGCA GGAATGCTTG CTGTCAACGC TGGTATTTTA
    CCTTTAATGG AGCTGAATGT TCAGGACCTC TTCCCATTGA AGCTATCATC TATCTGGACC
    AAGGAAGCCC TGAGTTAAAT TCAACTATTA ATATTCATCG TACTTCCTCC GTGGAAGGAC
    TCTGTGAAGG GATTGGTGCT GGACTGGTAG ACGTGGCCAT CTGGGTCGGC ACCTGTTCAG
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Figure 10

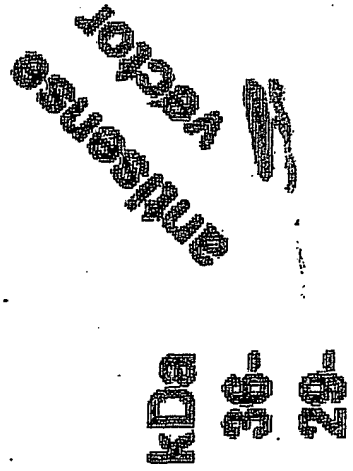


Figure 11

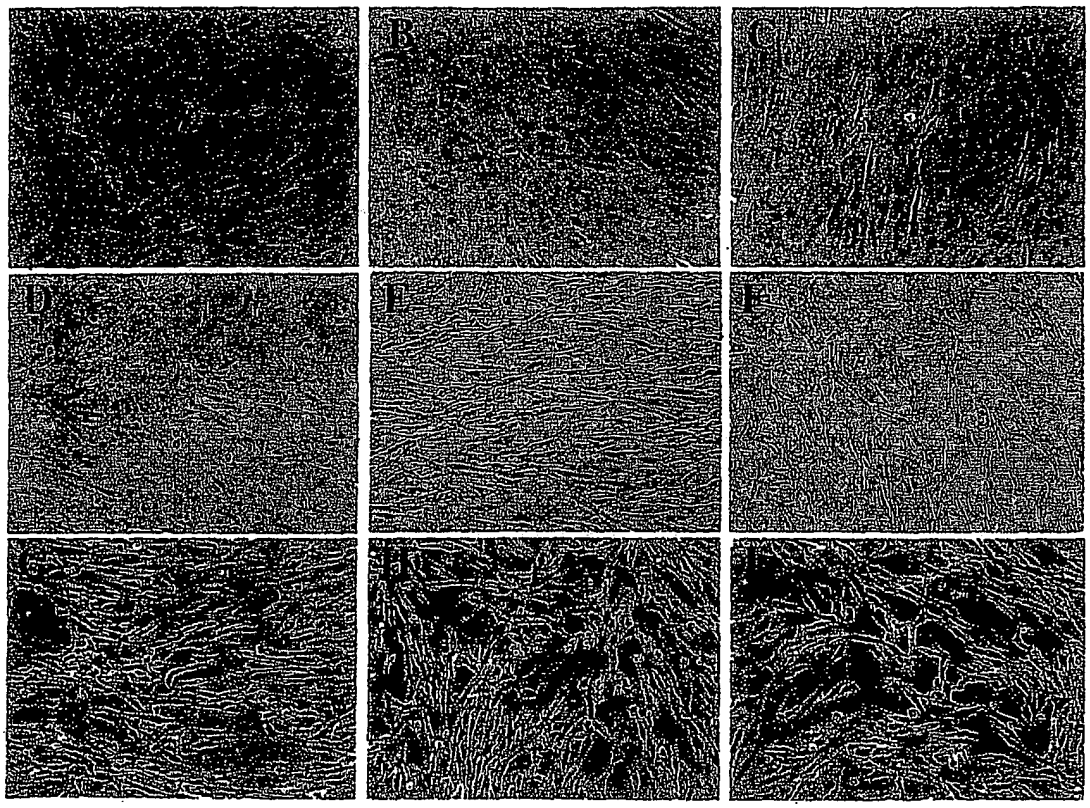
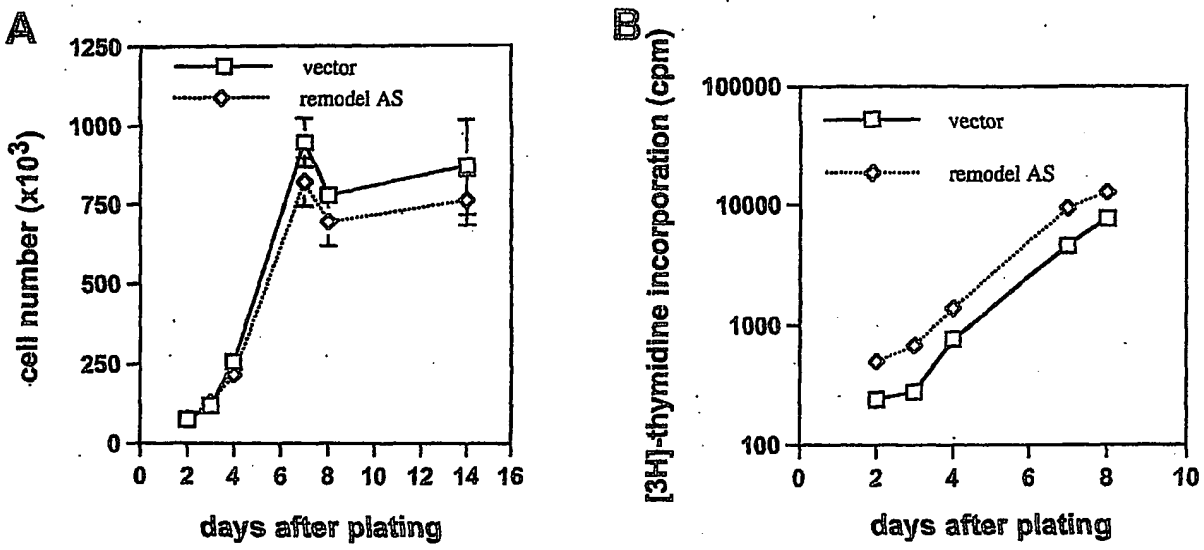


Figure 12


A
cell number (x10^3)
1250
1000
750
500
250
0
0 2 4 6 8 10 12 14 16
days after plating
vector
remodel AS
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100000
10000
1000
100
0 2 4 6 8 10
days after plating
vector
remodel AS

Figure 13

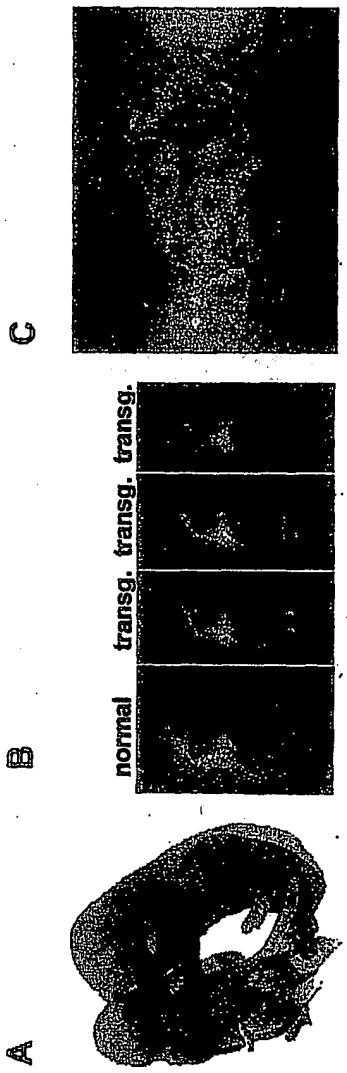
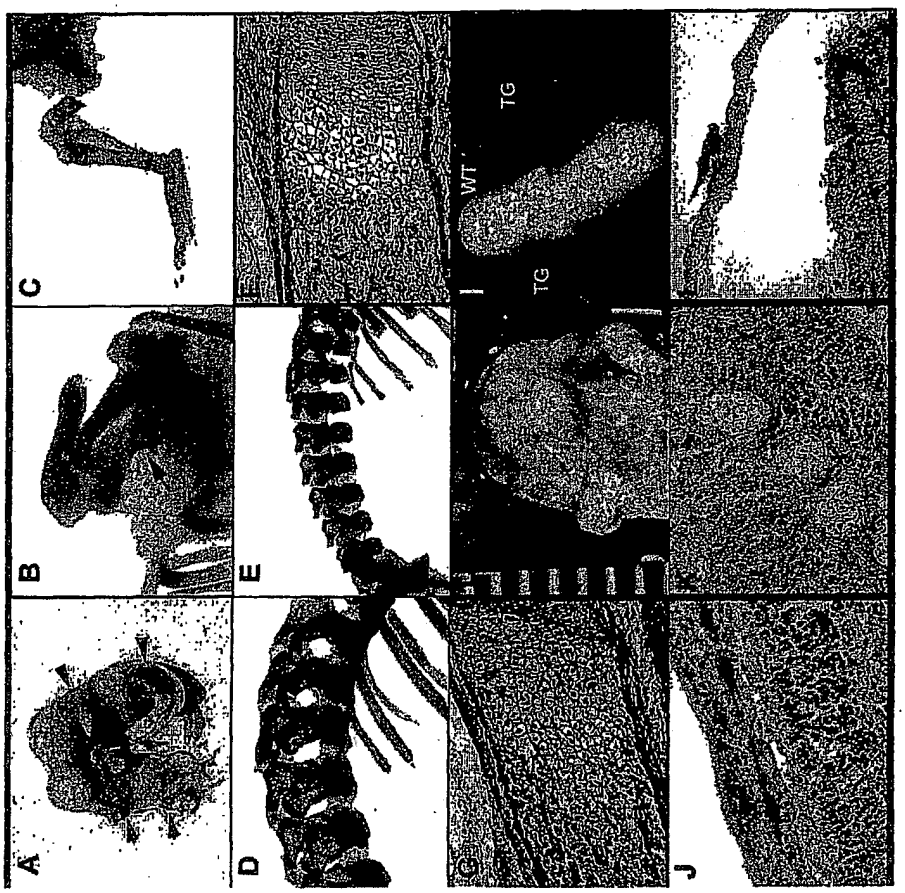
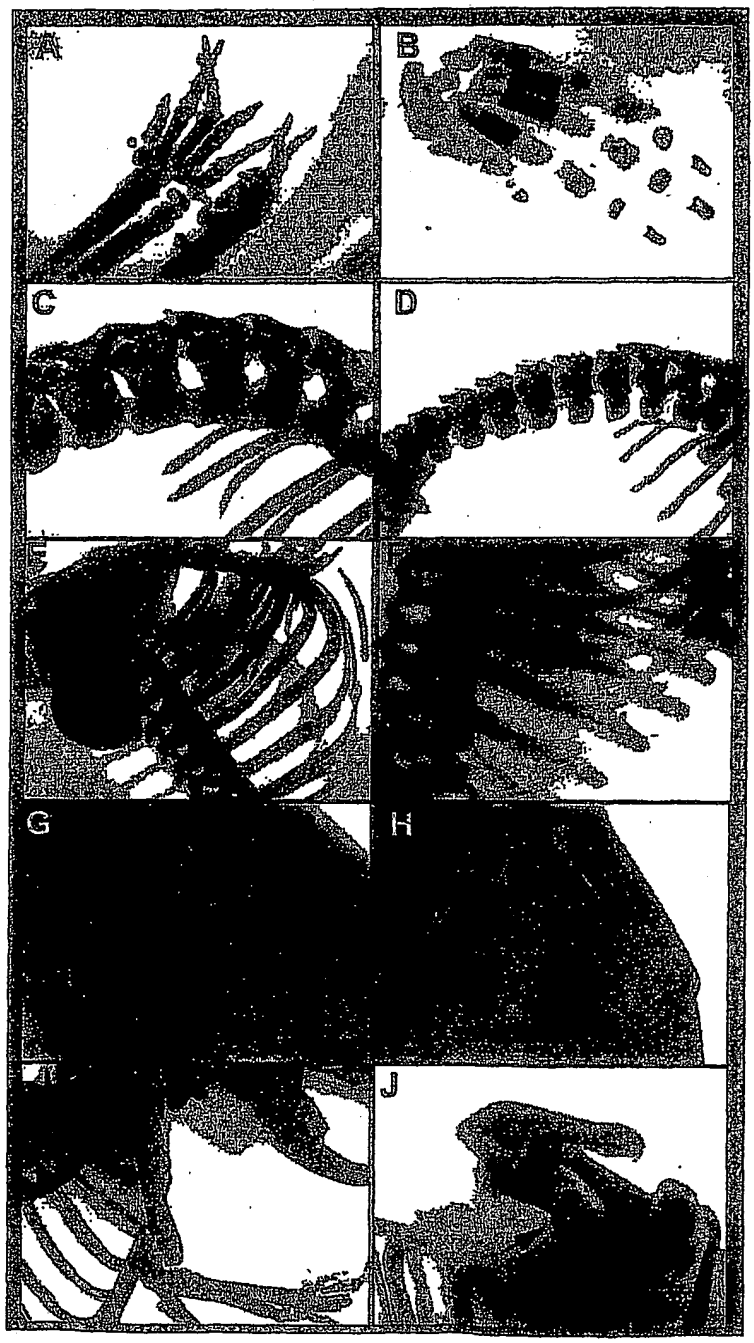


Figure 14

Figure 15





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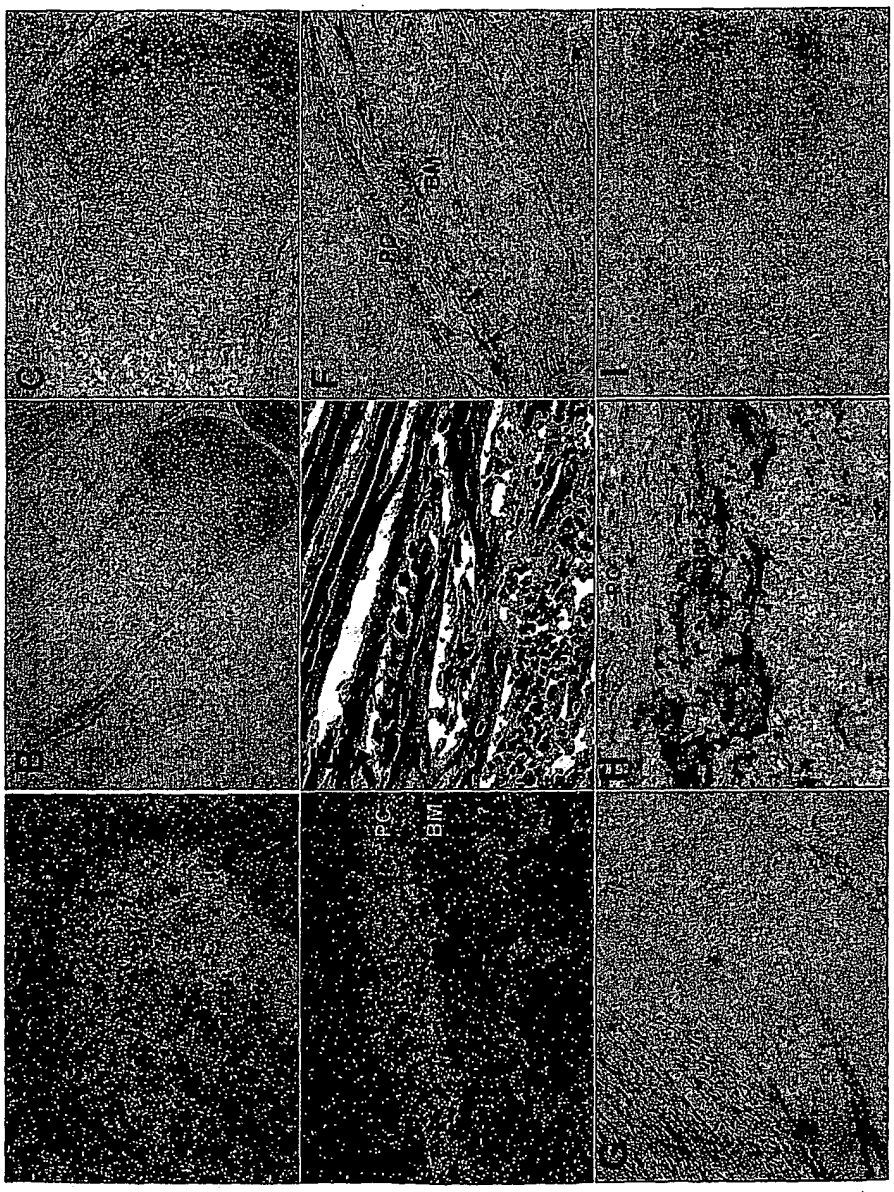

Figure 17

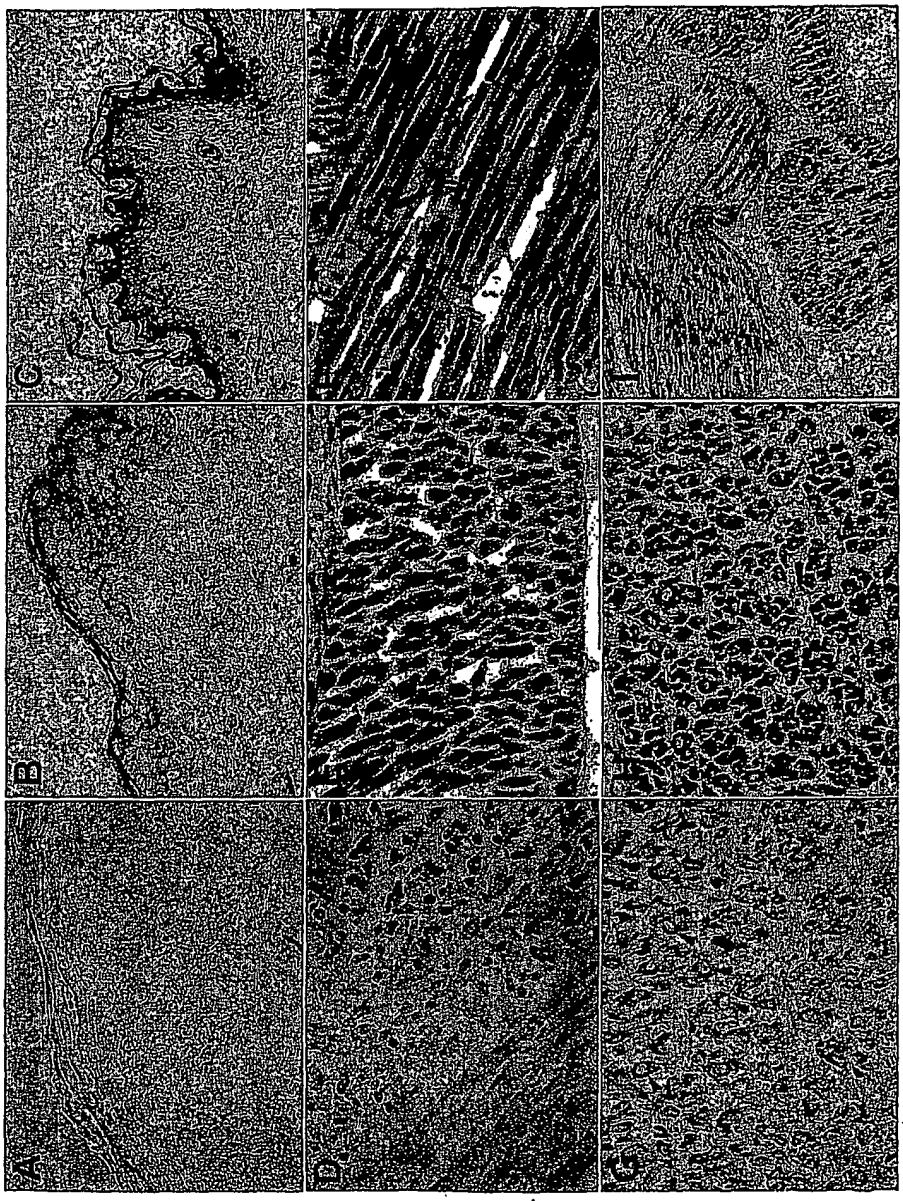

Figure 18

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AAUGGGAAGAGGUCCUGAACAUUCAGCUCCAUUAAAGGUAAAAUACCAGCGUUGACAG
CAAGCAUUCCUGCAUUUGAGCCGAAGCGAGCCACUGAACAGAACUCGAAGAGCGCUGU
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Figure 19

SEQUENCE LISTING

<110> MAINE MEDICAL CENTER RESEARCH CENTER
LINDNER, Volkhard
FRIESEL, Robert E.

<120> COMPOSITIONS, METHODS AND KITS RELATING TO REMODELIN

<130> 053689-5006-01

<150> US 09/692,081
<151> 2000-10-19

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Ser Glu Asn Pro Lys Val Lys Gln Lys Ala Leu Ile Arg Gln Arg Glu
35 40 45

Val Val Asp Leu Tyr Asn Gly Met Cys Leu Gln Gly Pro Ala Gly Val
50 55 60

Pro Gly Arg Asp Gly Ser Pro Gly Ala Asn Gly Ile Pro Gly Thr Pro
65 70 75 80

Gly Ile Pro Gly Arg Asp Gly Phe Lys Gly Glu Lys Gly Glu Cys Leu
85 90 95

Arg Glu Ser Phe Glu Glu Ser Trp Thr Pro Asn Tyr Lys Gln Cys Ser
100 105 110

Trp Ser Ser Leu Asn Tyr Gly Ile Asp Leu Gly Lys Ile Ala Glu Cys
115 120 125

Thr Phe Thr Lys Met Arg Ser Asn Ser Ala Leu Arg Val Leu Phe Ser
130 135 140

Gly Ser Leu Arg Leu Lys Cys Arg Asn Ala Cys Cys Gln Arg Trp Tyr
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Phe Thr Phe Asn Gly Ala Glu Cys Ser Gly Pro Leu Pro Ile Glu Ala
165 170 175

Ile Ile Tyr Leu Asp Gln Gly Ser Pro Glu Leu Asn Ser Thr Ile Asn

180 185 190

Ile His Arg Thr Ser Ser Val Glu Gly Leu Cys Glu Gly Ile Gly Ala
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Gly Leu Val Asp Val Ala Ile Trp Val Gly Thr Cys Ser Asp Tyr Pro
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35 40 45

Asp Leu Tyr Asn Gly Met Cys Leu Gln Gly Pro Ala Gly Val Pro Gly
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Arg Asp Gly Ser Pro Gly Ala Asn Gly Ile Pro Gly Thr Pro Gly Ile
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85 90 95

Ser Phe Glu Glu Ser Trp Thr Pro Asn Tyr Lys Gln Cys Ser Trp Ser
100 105 110

Ser Leu Asn Tyr Gly Ile Asp Leu Gly Lys Ile Ala Glu Cys Thr Phe
115 120 125

Thr Lys Met Arg Ser Asn Ser Ala Leu Arg Val Leu Phe Ser Gly Ser
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Leu Arg Leu Lys Cys Arg Asn Ala Cys Cys Gln Arg Trp Tyr Phe Thr
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Phe Asn Gly Ala Glu Cys Ser Gly Pro Leu Pro Ile Glu Ala Ile Ile
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Tyr Leu Asp Gln Gly Ser Pro Glu Met Asn Ser Thr Ile Asn Ile His

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Arg Thr Ser Ser Val Glu Gly Leu Cys Glu Gly Ile Gly Ala Gly Leu
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35 40 45

Phe Leu Val Leu Leu Leu Leu Leu Gln Leu Ser Ala Pro Ser Ser Ala
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Ser Glu Asn Pro Lys Val Lys Gln Lys Ala Leu Ile Arg Gln Arg Glu
65 70 75 80

Val Val Asp Leu Tyr Asn Gly Met Cys Leu Gln Gly Pro Ala Gly Val
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Pro Gly Arg Asp Gly Ser Pro Gly Ala Asn Gly Ile Pro Gly Thr Pro
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Gly Ile Pro Gly Arg Asp Gly Phe Lys Gly Glu Lys Gly Glu Cys Leu
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Arg Glu Ser Phe Glu Glu Ser Trp Thr Pro Asn Tyr Lys Gln Cys Ser
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Trp Ser Ser Leu Asn Tyr Gly Ile Asp Leu Gly Lys Ile Ala Glu Cys
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Thr Phe Thr Lys Met Arg Ser Asn Ser Ala Leu Arg Val Leu Phe Ser

165 170 175

Gly Ser Leu Arg Leu Lys Cys Arg Asn Ala Cys Cys Gln Arg Trp Tyr
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Phe Thr Phe Asn Gly Ala Glu Cys Ser Gly Pro Leu Pro Ile Glu Ala
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Ile Ile Tyr Leu Asp Gln Gly Ser Pro Glu Leu Asn Ser Thr Ile Asn
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Ile His Arg Thr Ser Ser Val Glu Gly Leu Cys Glu Gly Ile Gly Ala
225 230 235 240

Gly Leu Val Asp Val Ala Ile Trp Val Gly Thr Cys Ser Asp Tyr Pro
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auuaauaguu gaauuuaacu cagggcuucc uugguccaga uagaugauag cuucaauggg 180
aagagguccu gaacauucag cuccauuaaa gguaaaauac cagcguugac agcaagcauu 240
ccugcauuug agccgaagcg agccacugaa cagaacucga agagcgcugu uggaucgcau 300
cuuugugaau guacauuccg caauuuuccc aagaucuaug ccauaauuaa gugaacucca 360
ugaacacugc uuguaguuug ggguccagga uuccucaaag cuu 403

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<223> Description of Artificial Sequence:carboxy-terminal amino acids of REMODELIN

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<213> Artificial Sequence

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4

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(21) International Application Number:
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(25) Filing Language: English

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(30) Priority Data:
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(71) Applicant *(for all designated States except US)*: **MAINE MEDICAL CENTER RESEARCH INSTITUTE** [US/US]; 81 Research Drive, Scarborough, ME 04071-7205 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LINDER, Volkhard** [US/US]; 10 Sawyer Brook Circle, South Portland, ME 04106 (US). **FRIESEL, Robert** [US/US]; 28 Tall Pine Road, Cape Elizabeth, ME 04107 (US).

(74) Agents: **ALVAREZ, Raquel** et al.; Morgan Lewis & Bockius LLP, 1701 Market Street, Philadelphia, PA 19103 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
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26 February 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2002/042487 A3

(54) Title: COMPOSITIONS, METHODS AND KITS RELATING TO REMODELIN

(57) Abstract: The invention relates to novel nucleic acids encoding a mammalian adventitia inducible and bone expressed gene designated REMODEL, and proteins encoded thereby, whose expression is increased in certain diseases, disorders, or conditions, including, but not limited to, negative remodeling, arterial restenosis, vessel injury, ectopic ossification, fibrosis, and the like. REMODELIN also plays a role in cell-cell and cell-matrix adhesion, bone density, bone formation, dorsal closure, one mineralization, calcification/ossification, and is associated with *spina bifida*-like phenotype. In addition, the invention related to affecting REMODELIN expression by administration of TGF-ß and control of cellular gene expression using REMODELIN. The invention further relates to methods of treating and detecting these diseases, disorders or conditions, comprising modulating or detecting REMODELIN expression and/or production of REMODELIN.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/50940

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12P 21/06; C12N 15/87, 1/20, 15/12. 15/63
US CL : 536/23.5; 435/69.1, 445, 252.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.5; 435/69.1, 445, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, WEST, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CARNINCI et al. High-efficiency full-length cDNA cloning. Methods Enzymol. 1999, Vol. 303, pages 19-44.	1-3 and 19-20
X	WO200052151 A2 (INCYTE PHARMACEUTICALS, INC.) 08 Septemper 2000(08.09.2000), see SEQ ID NO: 27, claims 4-8, page 75.	1-3, 8-15, 19-20
Y	MATUNIS et al. SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. J Cell Biol. 09 February 1998 (09.02.1998), Vol. 140, No.3, pp.499-509. see pp. 500, under Plasmid Constructions.	6-7
Y	U.S. 4,761,406 A (FLORA et al) 02 August 1988 (02.08.1988), column 1, paragraph 1; column 2, paragraph 3 and columns 13-15.	34-41
A	Database GenBank, Accession No. AK003674. 05 December 2002 (05.12.2002).	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
14 July 2003 (14.07.2003)

Date of mailing of the international search report
05 NOV 2003

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Maher M. Haddad

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/50940

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3,6-15,19-20 and 34-41 (as they read on SEQ ID NO:1)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US01/50940

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Search Authority has found 24 inventions claimed in the International Application covered by the claims indicated below:

I. Claims 1-3, 6-15, 19-20 and 34-41, drawn to an isolated nucleic acid of SEQ ID NO:1 encoding rat REMODEL, fragment thereof; vectors, host cells and a kit.

II. Claims 1-3, 6-15, 19-20, and 34-41, drawn to an isolated nucleic acid of SEQ ID NO:3 encoding human REMODEL, fragment thereof; vectors, host cells and a kit.

III. Claims 4-5, and 21, drawn to an isolated polypeptide of SEQ ID NO:2.

IV. Claims 4-5, and 21, drawn to an isolated polypeptide of SEQ ID NO:4.

V. Claims 4-5, and 21, drawn to an isolated polypeptide of SEQ ID NO:5.

VI. Claims 16-18, drawn to an antibody against SEQ ID NO:2.

VII. Claims 16-18, drawn to an antibody against SEQ ID NO:4.

VIII. Claims 16-18, drawn to an antibody against SEQ ID NO:5.

IX. Claim 22, drawn to a non-human mammal comprising the isolated nucleic SEQ ID NO:1.

X. Claim 22, drawn to a non-human mammal comprising the isolated nucleic SEQ ID NO:3.

XI. Claims 23-24, drawn a method of treating a disease mediated by malexpression of REMODEL as it reads on SEQ ID NO: 1,

XII. Claims 23-24, drawn a method of treating a disease mediated by malexpression of REMODEL as it reads on SEQ ID NO: 3,

XIII. Claims 25-26, drawn to a method of diagnosing arterial resternosis.

XIV. Claim 27, drawn to a method of diagnosing negative remodeling.

XV. Claims 28, drawn to a method of diagnosing fibrosis.

XVI. Claims 29, 31 and 33, drawn to a method of identifying a compound that affects expression of REMODEL in a cell.

XVII. Claims 30 and 32, drawn to a compound identified by claim 29 or claim 31.

XVIII. Claim 42, drawn to a method of increasing REMODELIN expression in a mammal comprising administering a REMODELIN expression of increasing amount of TGF-β.

XIX. Claim 43, drawn to a method of reducing REMODELIN expression in a mammal comprising administering a REMODELIN expression reducing amount of TGF-β receptor type II.

XX. Claims 44-47, drawn to a method of affecting cellular gene expression in a mammal, comprising administering a nucleic acid encoding REMODELIN of SEQ ID NO: 1.

XXI. Claims 44-47, drawn to a method of affecting cellular gene expression in a mammal, comprising administering a nucleic acid encoding REMODELIN of SEQ ID NO: 3.

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XXII. Claims 48-49, drawn to a method of treating bone disease in a mammal in need of such treatment, comprising administering an inhibitor of REMODELIN expression.

XXIII. Claims 50-51, drawn to a method of treating cartilage disease in a mammal in need of such treatment, comprising administering an inhibitor of REMODELIN expression.

XXIV. Claims 52-55, drawn to a method of diagnosing a bone disease in a mammal in a sample.

The inventions listed as Group 1-24 do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of the Group.

The specific technical feature of the Group 1 invention is the isolated nucleic acid of SEQ ID NO:1 encoding rat REMODEL, fragment thereof; vectors, host cells and a kit claimed therein while the specific technical feature of the Group 2 invention is the isolated nucleic acid of SEQ ID NO:3 encoding human REMODEL, fragment thereof; vectors, host cells and a kit claimed therein.an

The specific technical feature of the Group 3 is the isolated polypeptide of SEQ ID NO:2.

The specific technical feature of the Group 4 is the isolated polypeptide of SEQ ID NO:4.

The specific technical feature of the Group 5 is the isolated polypeptide of SEQ ID NO:5.

The specific technical feature of the Group 6 is the antibody against SEQ ID NO:2.

The specific technical feature of the Group 7 is the antibody against SEQ ID NO:4.

The specific technical feature of the Group 8 is the antibody against SEQ ID NO:5.

The specific technical feature of the Group 9 is the non-human mammal comprising the isolated nucleic SEQ ID NO:1.

The specific technical feature of the Group 10 is the non-human mammal comprising the isolated nucleic SEQ ID NO:3.

The specific technical feature of the Group 11 is the method of treating a disease mediated by malexpression of REMODEL as it reads on SEQ ID NO: 1,

The specific technical feature of the Group 12 is the method of treating a disease mediated by malexpression of REMODEL as it reads on SEQ ID NO: 3,

The specific technical feature of the Group 13 is the method of diagnosing arterial resternosis.

The specific technical feature of the Group 14 is the method of diagnosing negative remodeling.

The specific technical feature of the Group 15 is the method of diagnosing fibrosis.

The specific technical feature of the Group 16 is the method of identifying a compound that affects expression of REMODEL in a cell.

The specific technical feature of the Group 17 is the compound identified by claim 29 or claim 31.

The specific technical feature of the Group 18 is the method of increasing REMODELIN expression in a mammal comprising administering a REMODELIN expression of increasing amount of TGF-β.

The specific technical feature of the Group 19 is the method of reducing REMODELIN expression in a mammal comprising administering a REMODELIN expression reducing amount of TGF-β receptor type II.

The specific technical feature of the Group 20 is the method of affecting cellular gene expression in a mammal, comprising administering a nucleic acid encoding REMODELIN of SEQ ID NO: 1.

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The specific technical feature of the Group 21 is the method of affecting cellular gene expression in a mammal, comprising administering a nucleic acid encoding REMODELIN of SEQ ID NO: 3.

The specific technical feature of the Group 22 is the method of treating bone disease in a mammal in need of such treatment, comprising administering an inhibitor of REMODELIN expression.

The specific technical feature of the Group 23 is the method of treating cartilage disease in a mammal in need of such treatment, comprising administering an inhibitor of REMODELIN expression.

The specific technical feature of the Group 24 is the method of diagnosing a bone disease in a mammal in a sample.

Since the special technical feature of the Group 1 invention is not present in the Groups 2-24 claims and the special technical feature of the Groups 2-24 inventions are not present in the Group 1 claims, unity of invention is lacking.